

VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

Edited by

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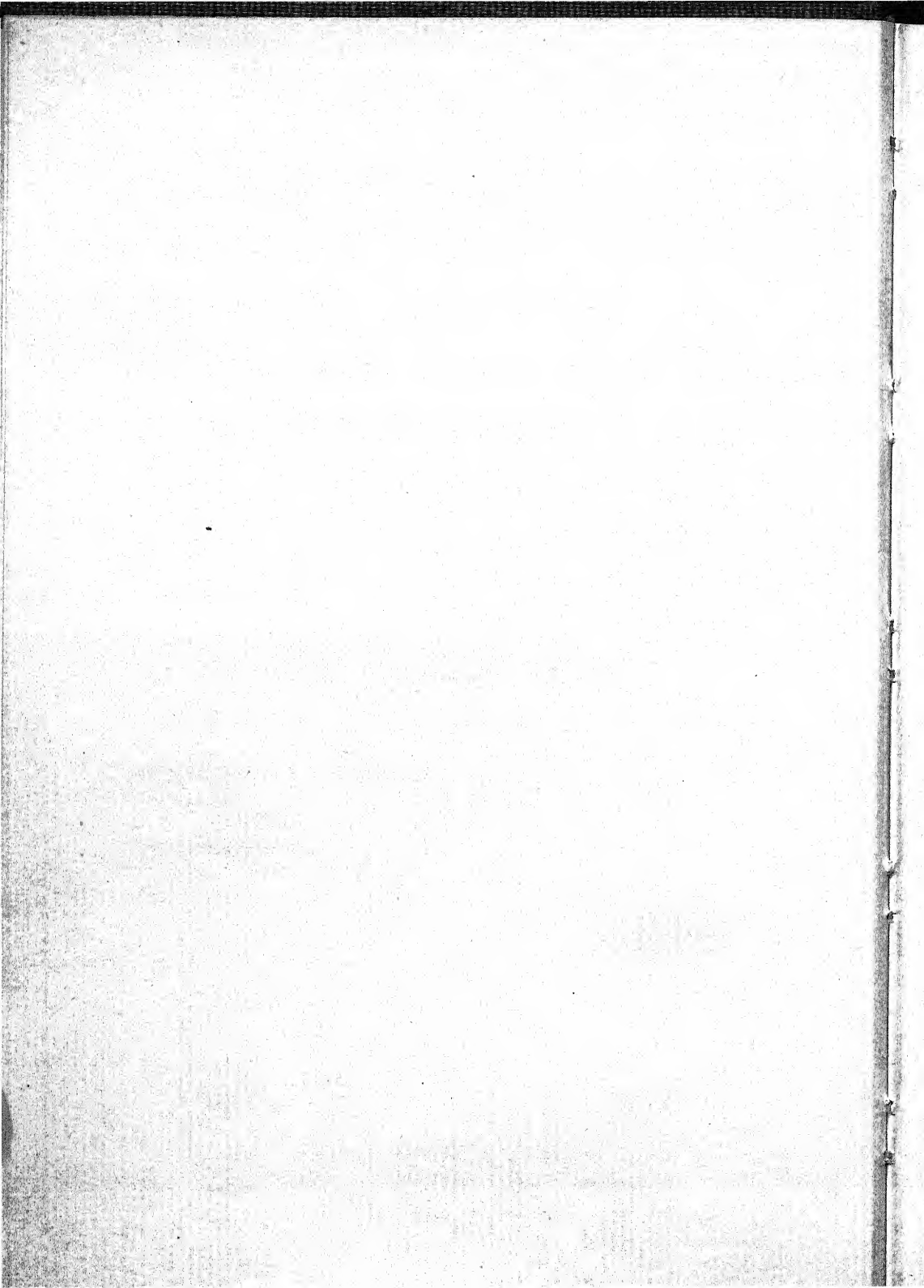
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Editors' Preface

The success of volume I of *Vitamins and Hormones*, in spite of the difficulties imposed by war conditions, has shown the need that exists for critical and intelligent reviews of this field. The scattering of the literature through a wide variety of journals, the inevitable variation in the quality of the work, and the variety of interests represented, ranging through physics and chemistry to pathology and clinical medicine, make the task of the reviewer a difficult one and the value of the review correspondingly greater.

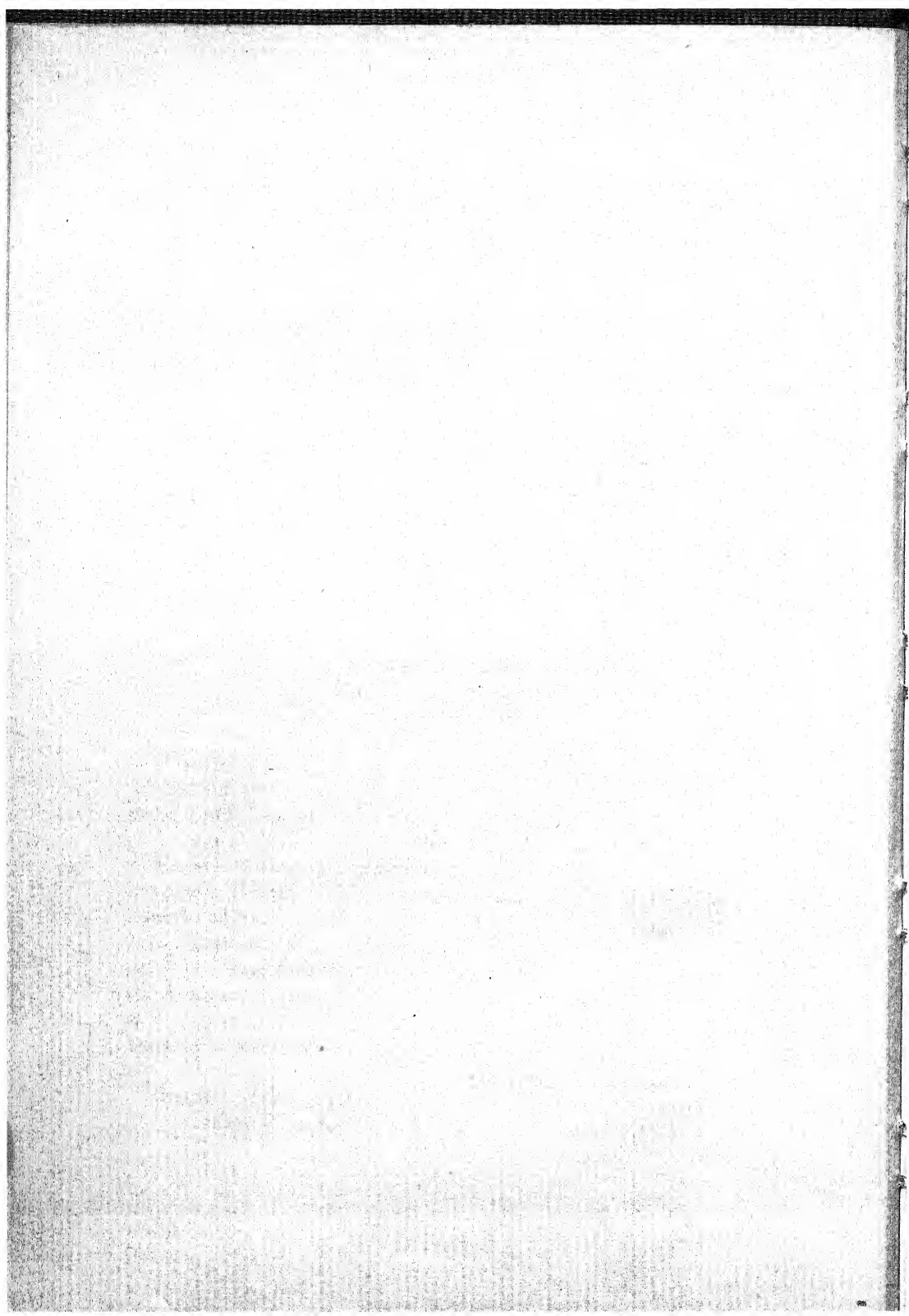
The Editors have felt that the authors should be free to exercise their judgment as to omission and selection of material, keeping in mind the aim of presenting a balanced picture of the development and current status of each subject. This is particularly true of fields of work which include numbers of inconclusive experiments. The two articles in the present volume relating to cancer provide an example of different approaches to this problem; in one the authors have aimed at discussing whatever work has been done, at the same time making clear the uncertain nature of some of it, while in the other only the best established results have been considered at all. Each author has difficulties of his own special kind to face, and the Editors wish to express their appreciation of the immense amount of work and thought which has gone into the preparation of all of the articles.

The policy of including complete subject and author indexes has been continued in the present volume, and it is believed that these indexes will add materially to the reference value of these yearly volumes. The Editors will welcome suggestions for improvement.

To some readers a volume like the present may appear to be essentially a series of complete but disconnected articles rather than an integrated whole. This must to a degree be true of any group of scientific reviews. In so far as it may be due to any real lack of correlation between ideas and results in different fields of endeavor, the bringing together of these reviews under one cover should be a stimulus toward closer interdependence of workers in distinct but related subjects. If such an integration is promoted, these volumes will have made a useful contribution to progress in the field of *Vitamins and Hormones*.

KENNETH V. THIMANN
ROBERT S. HARRIS

July, 1944



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The Role of Vitamins in the Anabolism of Fats

By E. W. McHENRY AND MADELEINE L. CORNETT

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I. INTRODUCTION

In the interpretation of the title of this review the authors have used Lusk's definition of anabolism (1): the construction of higher substances from lower ones. On this basis, the review deals with the possible effects of various vitamins upon the formation of complex lipids from much simpler molecules derived by metabolic processes from carbohydrates and proteins.

A number of recent reviews have dealt with the synthesis of lipids and with the effects of vitamins upon the synthesis and metabolism of lipids. Particularly valuable have been the excellent reviews by Longenecker (2), by Best and Lucas (3), by Bollman (4), and by Mitchell (5).

II. CLASSICAL DEMONSTRATIONS OF FAT FORMATION

During the past century the process of lipogenesis has been demonstrated repeatedly. Although Boussingault and Persoz had described fat formation in geese (6), Lawes and Gilbert (7) are responsible for the first evidence clearly indicative of such a process in animals. Analyses of carbohydrate, fat, and protein of the food consumed by pigs, as well as of the resultant composition of the carcasses, served to demonstrate that extensive formation of lipid material from carbohydrate and protein had taken place. These observations confirmed the opinion earlier expressed by Liebig (8) that fat could be synthesized in the animal body.

In 1886, Rubner (9) described lipogenesis in dogs and subsequently Rosenfeld (10) and also Morgulis and Pratt (11) contributed observations

on fat synthesis. The gaseous exchange experiments carried out by the latter, drew attention to the elevated respiratory quotient associated with active fat formation.

The synthesis of lipid material has also been shown to occur in bacterial and yeast cultures, in seeds, and in certain lower animals. In 1908, Weinland (12) described fat formation by macerated larvae incubated in a peptone medium. Beebe and Buxton (13) reached similar conclusions as a result of studies on *Bacillus pyocyaneus*; the pellicle formed by cultures of the organism after an incubation period of three weeks yielded a large amount of ether-extractable material.

More recent researches have indicated the extent to which this important metabolic process may function. In 1925, Wierzuchowski and Ling (14) corroborated the observation of Rapport and associates (15) on fat formation from carbohydrate by "well-nourished" swine. It was pointed out that lipid material was synthesized to the extent of 1% of the body weight daily. The contributions of Anderson and Mendel (16) on rats and of Ellis, *et al.* (17, 18) on hog fattening are in harmony with the earlier observations. Benedict and Lee (19), in a detailed study of fat formation in forced-fed geese, have emphasized the high degree to which lipogenesis may predominate in the metabolic processes of animals possessing a disposition to fat formation. Schoenheimer, *et al.* (20, 21) have demonstrated the rapidity with which lipogenesis occurs, and both Longenecker (22), and Hoagland and Snider (23) provided conclusive evidence of lipid formation from amino acids.

Although it had often been suggested that fat synthesis is essentially, if not exclusively, a hepatic function, only rather recently has satisfactory experimental support for this view become available. Bernhard and Schoenheimer (21) drew attention to the extreme rapidity of turnover of liver fatty acids, and Winter, *et al.* (24) described the failure of fat synthesis in Eck fistula dogs. Similar conclusions may be drawn from experiments of Barrett, Best, and Ridout (25), McHenry (26), and Stetten and Schoenheimer (27).

It is of interest that Hildesheim and Leathes (28) reported the formation of fat in minced dog, rabbit, and pig livers *in vitro*. However, their results were not considered conclusive since added carbohydrate did not stimulate lipid accumulation.

In investigations prior to 1910 and, indeed, in many subsequent to this date, no attention was paid to the vitamin content of the experimental diets. More recent investigations have indicated the essential relations of some vitamins to fat synthesis and have explained why certain foods proved to be lipogenic in the earlier work.

III. THE SYNTHESIS OF FAT FROM CARBOHYDRATE

In 1936, Whipple and Church (29) extended observations made previously by Sure and associates (30, 31, 32), by Graham and Griffith (33), and by Mitchell (34) that vitamin B₁ had a specific effect upon body weight, as determined by using isocaloric feeding. In addition to repeating the earlier observations upon body weight, Whipple and Church analyzed the animal bodies and secured information regarding the reasons for the increase in weight. They found that, of the difference in weight between pair fed rats, 51% was due to deposition of fat and the balance to water. Since the animals had been maintained on a fat-free diet, and since the only difference in treatment of the two groups was the supply of thiamin to one group, Whipple and Church concluded that thiamin plays a rôle in the synthesis of fat in the animal body. In the following year, they reported (35) a marked difference in respiratory quotient between two isocalorically fed groups of rats, one of which received thiamin; the latter had a quotient well above unity, thus strengthening the hypothesis that thiamin brings about fat formation.

The observations regarding fat formation and deposition were confirmed by McHenry in 1937 (26), who also suggested that the effect of thiamin on fat synthesis could be harmonized with observations on the effect of the vitamin upon pyruvate utilization (36). In 1938, McHenry and Gavin began a series of reports on the relation of B vitamins to fat synthesis and metabolism. Thiamin was also found to cause synthesis in pigeons (37). Riboflavin and rice polish concentrate, used as a source of the B complex, given with thiamin, augmented the amount of synthesized fat (38). Pyridoxin, supplied with thiamin and riboflavin, likewise had a supplementary effect (39). In studying the effects of other members of the complex, an extract of beef liver was used as a source of unknown factors; this markedly augmented the amount of fat and caused fatty livers containing increased amounts of cholesterol (40). These fatty livers were not prevented by choline but were prevented by lipocaic (41). Later it was shown that biotin had a similar effect to that of the liver fraction (42) and that inositol acted like lipocaic in preventing the fatty livers caused by biotin in rats (43).

Included in the data arising from work with biotin, McHenry and Gavin gave results (42) which showed a specific effect of thiamin upon fat synthesis but to which they failed to call attention. The amounts of fat produced by various combinations of known B vitamins were cited. Riboflavin, pyridoxin, and pantothenic acid had augmentory effects upon fat synthesis. Examination of the data shows that biotin, while causing fatty livers, actually did not increase the percentage of body fat (as compared with the amount of body fat produced by all of the isolated B vita-

mins except biotin). The data show that rats given biotin were heavier and contained a larger total weight of fat but that the percentage of body fat was smaller. It is also clear from the data that no synthesis of fat occurred when thiamin was omitted from the supplements, even though five other B vitamins were supplied. The results obviously indicated that thiamin is essential for the synthesis of fat from carbohydrate and that other members of the B complex can augment the amount of synthesis.

Longenecker, *et al.* (44) studied the composition of the fats synthesized by the action of thiamin. Rats were depleted for three weeks on a diet free of B vitamins and of fat. At the end of this period one half of the number of animals were killed and tissue analyses made; the remaining rats were continued on the basal diet plus thiamin for twelve days, when they were killed and used for analysis. During the depleting period, body stores of fat were utilized and apparently no synthesis occurred; there was a marked increase in the iodine number of both liver and body fats. Thiamin administration caused a large increase in the amount of fat and a marked drop in iodine number; liver and body fats of supplemented animals contained a high proportion of C_{16} acids, a finding which Longenecker had previously shown (45) to be characteristic of synthesized fat. In a later study the composition of liver and body fat as effected by other B vitamins, by liver fraction (as a source of biotin), and by lipocaic was investigated (46). Fatty acids synthesized when thiamin, riboflavin, pyridoxin, and choline were given were largely C_{16} and C_{18} acids, the C_{16} acids being 54% of the total. Further supplementing the diet with the liver fraction caused a greater increase of C_{18} acids than of C_{16} acids and also augmented the quantity of unsaturated acids. This change in the fatty acid composition, and particularly the increase in unsaturated acids is of interest in connection with the question of essential fatty acids; in most of the studies on the latter subject, yeast has been used as a source of the B vitamins. It may well be that a concentration of the vitamins, different from that supplied by yeast, might cause a synthesis of essential fatty acids.

The hypothesis that thiamin has a specific effect upon fat synthesis has been criticized by Quackenbush, Steenbock, and Platz (47). Data from two types of experiments were cited in support of the criticism. In the first case, three groups of rats were used, all of which had been depleted on a diet containing autoclaved yeast, the first group was killed and analyzed at the end of the depleting period, the second group received thiamin and the basal diet *ad libitum*, the third group were given the same amount of thiamin as used in the second group but the intake of the basal diet was so restricted that body weights were held stationary. At

the end of the depleting period, there was a marked reduction in the amount of fat and an increase in the iodine number. The second group showed a large increase in body fat and in body weight while the third group evidenced no increase in fat. Obviously, if food intake is restricted so as to maintain a constant body weight, there could be no increase in body fat unless other constituents, *e.g.*, protein or water, were diminished simultaneously. Restrictions of food intake on this basis would, of course, prevent any evidence of fat synthesis as shown by an increase in body fat. These results should be contrasted with those obtained by the use of isocaloric feeding. There have been repeated demonstrations of the specific effect of thiamin upon body weight and body fat when isocaloric feeding was used (26, 29). In such experiments the effect of thiamin upon metabolism is clearly evident. In the second type of experiment Quackenbush, *et al.* used a basal diet containing thiamin and riboflavin but designed to produce acropdynia. After maintenance on this diet for five weeks, one group of rats was killed for analysis, a second received extra thiamin for three weeks, a third was given a rice bran concentrate, and a fourth, with the same amount of rice bran concentrate, had the intake of the basal diet restricted so that a constant body weight was maintained; after these supplements had been given for three weeks the animals were killed for analysis. The first group showed only a small amount of body fat, and the authors interpreted this result as indicating that a continuous supply of thiamin had not prevented a loss in body weight nor accomplished fat formation. However, the iodine numbers of the liver and body fats were significantly lower than was the case in the group of animals not given thiamin in the first experiment, suggesting that fat synthesis had taken place. It is possible that, in the acute stages of pyridoxin and pantothenic acid deficiencies, fat is rapidly utilized and disappears soon after formation. Deficiencies of these vitamins were certainly more severe than had been obtained in the work of Gavin and McHenry (42). The question of the extent of deficiencies of these two vitamins was raised by Quackenbush, *et al.*; Gavin and McHenry had produced deficiencies of these vitamins to such an extent that animals responded readily to supplements but the criticism of this aspect appears to be partially justified. The third group of animals, receiving rice bran concentrate, showed a marked increase in fat. Quackenbush, *et al.* stated that this showed that a correction of any deficiency would bring about fat synthesis. However, they did not try the effects of pyridoxin nor of pantothenic acid in the absence of thiamin; under this condition it has been shown that fat formation does not occur (42). Indeed, a supply of four B vitamins to deficient animals will not cause fat synthesis *unless* thiamin is furnished (42). The additive effect of other B vitamins had been shown, previously to the

work of Quackenbush, *et al.*, by Gavin and McHenry in connection with riboflavin (38), rice bran concentrate (38), pyridoxin (39), pantothenic acid (42), and biotin (42).

In studies on the relation of the B vitamins to fat synthesis and metabolism the effects of lipotropic agents must be considered. For example, if thiamin is given as the only supplement to a basal diet rich in carbohydrate and low in protein, there will be a marked increase in liver fat (26). This increase can be prevented by supplying choline or a choline precursor, such as a large amount of casein. Choline can be inadvertently furnished. In the investigation of Engel and Phillips (48) fatty livers were obtained, but not acutely so because the basal diet contained peanuts and yeast, both good sources of choline. Quackenbush, Steenbock, and Platz (47) noted that fatty livers were not secured in their animals, even with thiamin supplementation. However, both of their experimental diets provided 18% casein, an amount sufficient to be lipotropic, and one of the diets contained two sources of choline: autoclaved yeast and starch.

In a recent paper, Stetten and Grail (49) have described studies on fat synthesis with the use of deuterium. The effects of pyridoxin and pantothenic acid in augmenting fat synthesis were confirmed, but these authors, like Quackenbush, *et al.*, did not give these vitamins without thiamin. As has been pointed out previously the addition of other B vitamins not only increases the amount of fat but also causes a change in the character of the fat. Reports by Engel (50), by Forbes (51) and by Gavin, *et al.* (52) have shown that the supply of various B vitamins alters the lipotropic effect of choline. This may partially explain the observation by Stetten and Grail regarding the very slight effect of choline in their experiments.

Longenecker, *et al.* (44) found that thiamin, as the only supplement, caused a marked synthesis of saturated C_{16} acids. If the diet is deficient in choline, or its precursors, fats accumulate in the liver; this can be prevented by a supply of choline. McHenry and Gavin (42) reported the production of fatty livers, by adding biotin and other B vitamins to the diet, which were resistant to the action of choline. Engel (50) has found that thiamin, riboflavin, pyridoxin, and pantothenic acid produce a choline-resistant type of fatty liver. Under this condition choline and inositol, given simultaneously, will maintain liver fat at a normal level. Engel believed that pyridoxin was concerned with this effect since small amounts of choline were effective in animals depleted of pyridoxin for eight weeks or longer. A different result was obtained by Forbes (51), who found that choline was less effective in rats receiving nicotinic acid. These findings of Engel and of Forbes again raise the point regarding alterations in the type of fatty acids synthesized by changes in the B vitamin supplements. This deserves further study. When thiamin is the only B vitamin supplied, the character

of the fat synthesized and deposited in the body is radically different from that found in "normal" animals. The question as to which B vitamins should be given to animals to cause the production of a more "normal" type of body fat is a matter for further investigation. It may well be that the quantitative proportion of the B vitamins may be a factor, influencing the character of the synthesized fat.

Some attention has been given to investigation of possible effects of other dietary factors in fatty acid synthesis or retention. Sheppard and McHenry (53) reported that the fat content of vitamin-C-deficient guinea pigs was greatly reduced in comparison with that of normal animals and that pair-fed guinea pigs receiving ascorbic acid contained even less fat than the members of the deficient group. In these experiments, ascorbic acid did not apparently cause fat synthesis. However, experiments carried out by Patterson, McHenry, and Crandall (54) indicated an effect of vitamin A upon either fat synthesis or retention. Using a diet which provided only a very small amount of lipid material, pair-fed rats receiving carotene contained more fat than did the deficient control animals and practically as much as rats fed *ad libitum*. These results were in agreement with observations reported in 1932 by Sampson and Korenchevsky (55). These authors had also noted the higher fat content of pair-fed rats receiving vitamin A. It is not clear as to whether the increase in fat content was due to synthesis and further investigations are needed.

IV. THE SYNTHESIS OF FAT FROM PROTEIN

After reviewing evidence regarding the formation of fat from protein, Mitchell and Hamilton (56) stated in 1929, "of all the possible transformations of nutrients in the animal body, that of the conversion of protein into fat, or, more correctly, of amino acids into the higher fatty acids entering into the composition of animal fats, has probably attracted the greatest amount of experimental enquiry and occasioned the most controversy. . . . Purely on chemical grounds, the conversion of protein to sugar to fat, involving a complicated and uneven series of cleavages, oxidations, and condensations, would seem to be a clumsy and thoroughly inefficient method of disposing of an excess of dietary protein." After considering the demonstrations of fat synthesis from protein in bacteria by Bævre and Buxton (13), and in mammalian eggs by McClendon (57), Mitchell and Hamilton concluded that, while a clear-cut proof of fat formation from protein had not yet been provided in higher animals, a considerable probability that such a transformation does occur under certain conditions had been established.

While the work of Lawes and Gilbert (7) is generally cited (2) as the first proof of fat formation from carbohydrate, a careful examination of their data shows that a reasonable amount of the fat synthesis which they ob-

served must have been obtained from protein. The first completely convincing evidence of the ability of higher animals to convert protein to fat was furnished by Longenecker in 1939 (22) and confirmed by Hoagland and Snider in the same year (23). In both cases rats were maintained on diets rich in casein but free of fat and carbohydrate. On such a diet Hoagland and Snider secured a gain in body weight of 67% and an increase of body fat of 257%. The procedure used by Longenecker is of interest because it has been used by other workers and has been criticized. A loss in body weight of about 25% was produced in rats by inanition, and the animals were then fed a special diet until weight was restored. The special diet used to demonstrate the synthesis of fat from protein contained 96% casein, was free from carbohydrate or fat, and B vitamins were supplied in yeast. It was found that large amounts of fat were synthesized from protein and that, consequently, the body weights of the rats were increased. Longenecker gave data regarding the composition and characteristics of the synthesized fat. While the inference was drawn that fat from protein was quite similar to that obtained from carbohydrate, the data show some differences. These are worth noting because it would be assumed that, if fat is formed from protein through an intermediate carbohydrate stage, this fat should be similar to that synthesized from carbohydrate supplied in the diet. "Protein-fat" contained a larger proportion of saturated fatty acids than did "carbohydrate-fat"; in terms of some individual fatty acids, "protein-fat" contained more palmitic and arachidic acids and less oleic acid. The conclusion that "protein-fat" is entirely similar to "carbohydrate-fat" was drawn by Eckstein in 1929 (58). In this connection reference should again be made to the statement by Mitchell and Hamilton that it is unlikely that the synthesis of fat from protein proceeds by way of carbohydrate. However, the character of the fat synthesized from either carbohydrate or protein may depend upon the vitamin supplements.

At least three types of evidence have indicated that one or other members of the vitamin B complex are concerned with protein metabolism. In 1927, Hasson and Drummond (59), and in 1928, Hartwell (60) showed that an increased supply of the B vitamins was necessary for normal increase in weight when high-protein diets were fed; Hasson and Drummond indicated that two factors, one of which was heat-stable, were essential for protein metabolism. Investigations by Richter and Hawkes in 1940-1941 (61, 62) on the choice of foodstuffs by rats depleted of several members of the B complex also indicated a relationship between proteins and these vitamins.

In 1940, McHenry and Gavin (63), using a procedure patterned after that of Longenecker, studied the effects of various B vitamins upon the synthesis of fat from protein. Adult rats, about 200 g. in weight, were placed for three weeks upon a diet free of fat and of the B vitamins. During

this depleting period the amount of body fat was sharply reduced, and there was a loss in body weight of about 25%. After this period the animals were fed a diet containing 96% casein but no fat nor carbohydrate; B vitamins were given singly and in various combinations. This treatment was continued for 10 days when the rats were killed and fat determinations made. Only in those animals which received thiamin and pyridoxin was there any evidence of fat formation and consequent increase in body weight. When (thiamin, riboflavin, pantothenic acid, nicotinic acid, and choline were all given, the loss in body fat and body weight, characteristic of the depleting period, was continued throughout the supplemental period. In contrast, animals which received the same supplements plus pyridoxin showed a three-fold increase in body fat. On the basis of these observations it was concluded that pyridoxin is concerned with protein utilization.

In an extension of these observations Cornett and McHenry (unpublished data) have shown that pyridoxin and thiamin are also essential for the formation of glycogen from protein. They found that the addition of biotin to the supplements, which, in the case of high carbohydrate diets, quickly causes the production of fatty livers containing large amounts of cholesterol, fails to do so with protein-rich diets. These preliminary results suggest that the path for the synthesis of fat from protein does not proceed by way of carbohydrate; otherwise, one would expect a similar result to that secured with a carbohydrate diet.

A vigorous criticism of this recent work on the relationship between the B vitamins and fat synthesis from protein has been made by Mitchell (64) and repeated by him more recently (5). After an incomplete description of the procedure employed by McHenry and Gavin, a description which omitted a fundamental feature, Mitchell stated:

"Only when pyridoxin was fed was the fatty acid content of the carcass appreciably greater than that of the controls, but only in such cases did any growth occur. However, fat deposition is a necessary concomitant of growth and, regardless of the nature of the dietary characteristics that induce variable intakes of food, there will in general be a close correlation between the rate of growth secured and the fat content of the carcasses produced, within the range of submaximal rates of growth. For this reason, for instance, the supplementation of a ration deficient in sodium or chlorine by sodium chloride might be expected to produce, not only more rapid gain in growing rats, but also greater contents of carcass fat, without any implication that either sodium or chlorine is at all intimately involved in fat synthesis. Such technic cannot be expected to yield evidence of a direct and fundamental relationship between any dietary supplement and the synthesis of body fat."

It may be assumed that the essential nature of Mitchell's criticism is that

rats on a deficient diet do not grow as well as animals on an adequate diet and that the latter rats, because of better growth, will always contain more fat. This viewpoint is based, when used as a criticism of the results of McHenry and Gavin, upon an erroneous and misleading conception which is unfortunately evident in many papers on animal nutrition. For some years it has been the custom to plot animal weights and to assume that the result is a "growth" curve. Many dietary constituents, particularly the vitamins, have consequently been described as essential for growth. The fallacy of this misuse of the word "growth" was clearly pointed out by Orr and Richards in 1934 (65) but the fundamental point raised by them has been completely ignored by many investigators in the field of nutrition. Orr and Richards showed that, in rats deprived of vitamin A, the body weight reaches a maximum and then decreases, but true growth as measured by body length actually continues. As "growth" is interpreted in the current literature, rats in acute vitamin A deficiency should be described as exhibiting negative growth, a description which would be patently absurd but no more fallacious than the use of body weight as an index of growth. Fortunately, this misconception has not been used with humans; if it were, there would be the interesting illustration of a man showing two, or perhaps more, periods of growth during life, one during childhood and adolescence, and another in middle age. The use of weight as an index of growth in animal experiments is not only misleading and inaccurate but has actually handicapped advance in knowledge of the relation of the vitamins to metabolism. The time has surely arrived when workers in nutrition should use the simple word growth correctly.

In the case of the investigation criticized by Mitchell, emphasis on the misuse of the word growth is particularly pertinent. Adult rats were depleted for three weeks, during which time there was a marked loss in weight (but not negative growth). The effects of various supplements were then tried with the results reported above. Because there was a synthesis and deposition of fat, and perhaps of other substances, the body weight was, of course, increased. Since body length was not measured it is impossible to say whether growth also occurred. It does not always follow, as Mitchell assumed, that an increase of fat is concomitant with an increase in body weight. Sheppard and McHenry reported in 1939 (53) that guinea pigs receiving ascorbic acid but isocalorically fed with deficient controls are significantly heavier but contain much less fat than the deficient animals. In pointing out that any dietary supplement, by increasing weight, would also increase body fat, Mitchell used as an example either sodium or chlorine. In 1937 Kahlenberg, Black, and Forbes (66) reported that sodium supplementation enabled rats to synthesize fat. This was concluded from results of fat analysis of isocalorically fed animals. Unfortunately, no clear

conclusion as to fat synthesis was possible since the basal diets contained variable amounts of fat and the difference in fat intake between the two groups more than accounted for the difference in fat deposition.

A number of studies of body composition of rats of different ages have been made but in each case growth has been determined by body weight. An interesting study of the data from one investigation can be made. Pickens, Anderson, and Smith (67) reported the results of analyses of rat bodies at different ages. It has seemed advantageous to combine their data with figures for body length given by Donaldson (68). At an age of 100 to 120 days, curves for total ash, for protein, and for body length show plateaus while body weight and body fat continue to increase. Apparently, at that age, true growth ceases but the weight continues to be increased by a "fattening" process. A failure to distinguish between growth and changes in body weight and an interpretation that animals became heavier simply because of some mysterious growth process diverted the attention of investigators from fundamental studies on metabolism and delayed progress in the vitamin field. A good example of this was the important and useful report by Mitchell in 1933 (34) regarding the effect of vitamin B in causing a weight difference between rats fed isocalorically. Data were available to focus attention upon the relation of thiamin to fat synthesis but, unfortunately, this was not done until the question was examined by Whipple and Church three years later (29).

Additional evidence that pyridoxin is concerned with protein metabolism was supplied by Foy and Cerecedo (69), who showed that a deficiency of this vitamin is much more rapidly produced in rats by maintaining them upon a protein-rich diet. Pyridoxin has not yet been linked to a particular enzyme system and further investigations will be needed to elucidate the rôle played by pyridoxin in protein degradation.

V. THE FORMATION OF PHOSPHOLIPIDS

So far as can be ascertained, no effect of vitamins upon the synthesis of phospholipids has been described, unless choline is considered to be a vitamin. While such a classification of choline seems to be unnecessary, a number of writers have done so, and a brief reference to this substance should be included. For a detailed discussion of choline, recent reviews by McHenry (70), by Griffith (71) and by Best and Lucas (3) may be consulted. The anabolism and function of the phospholipids has recently been reviewed by Sinclair (72).

The demonstration that choline would prevent the formation of some types of fatty livers led to several explanations of its lipotropic action. Of these, the most plausible one, for which evidence was first provided by Welch (73), is that choline is lipotropic because it promotes the formation

of phospholipids. Perlman and Chaikoff (74) have furnished confirmatory evidence for this assumption. Choline is most effective as a lipotropic agent when fat accumulation in the liver consists mainly of simple glycerides and, more particularly, when the fatty acids are largely C_{16} or C_{18} , with a low iodine number. The activity of choline is influenced by several B vitamins (54); this may be due to a change in the character of the fatty acids present in the liver. The lipotropic effect of choline is also inhibited by the presence of large amounts of cholesterol (54). It may be that phospholipids can only be formed by the action of choline from certain fatty acids and not from an indiscriminate assortment.

Inositol has been shown by Gavin and McHenry (43) and by Engel (50) to have a lipotropic action. Inositol is present in considerable amounts in some naturally occurring phospholipids (75), and it is possible that inositol, like choline, owes its lipotropic activity to participation in the formation of phospholipids. There is no direct experimental evidence for the correctness of this hypothesis. It is of interest that inositol exerts its influence under conditions unfavorable for the action of choline, particularly in the presence of large amounts of cholesterol. Analysis of the phospholipids formed by the action of choline and of inositol would provide useful information regarding the mode of action of these two substances.

VI. THE SYNTHESIS OF CHOLESTEROL

The ability of the animal body to synthesize cholesterol was clearly shown by Channon (76), by Randles and Knudson (77), by Eckstein and Treadwell (78), and by Schoenheimer and Breusch (79). Schoenheimer and Breusch showed that mice maintained on a diet of bread, or of bread and fat, synthesized as much cholesterol in a month as was present in their bodies at the start of the experiment. Their work indicated that there is continuous formation and destruction of cholesterol and also that the administration of large amounts of fat has no significant effect upon cholesterol synthesis.

The nature of the metabolic precursors of cholesterol has been somewhat uncertain. Eckstein and Treadwell (78) believed that the use of a high fat diet caused increased formation of cholesterol. Rittenberg and Schoenheimer (80) concluded that cholesterol is formed by the coupling of a large number of small molecules. Their observations failed to support a possible conversion of fatty acids to sterol directly, but they stated that their results could be explained by the theory that cholesterol plays a rôle in the transport of fatty acids in the organism and that the handling of a larger amount of fatty acids may require the presence of a larger amount of cholesterol, the latter being formed according to the need.

In 1937, Sonderhoff and Thomas (81) reported that yeast, grown in a

medium containing deuterioacetate, had so great a deuterium content in the unsaponifiable portion that it seemed necessary to postulate the conversion of acetic acid to sterols. Bloch and Schoenheimer (82) and Bloch and Rittenberg (83), also using deuterioacetic acid, demonstrated that mice are able to synthesize cholesterol from acetate. This confirmed the previous report from Schoenheimer's laboratory that cholesterol is formed by the union of a number of small molecules (80).

In 1940, McHenry and Gavin (40, 41) reported that the feeding of a beef liver fraction, used as a source of unisolated components of the vitamin B complex, caused a marked increase in liver and body cholesterol in rats. It was stated that this increase was due to synthesis, since the basal diet was practically devoid of cholesterol. This observation was confirmed by Longenecker, *et al.* (46). Gavin and McHenry (42) later found that an effect, similar to that produced by the liver fraction, could be obtained by the administration of biotin in conjunction with thiamin, riboflavin, pyridoxin, and pantothenic acid. However, they pointed out that biotin did not increase the amount of cholesterol synthesis but did augment the amount of cholesterol in the liver. The increase in liver cholesterol is either the cause of, or accompanies, the production of a type of fatty liver which is not prevented by choline but which is prevented by lipocaic or by inositol. The data showed that the increase in liver cholesterol did not precede, but paralleled, the increase in liver fat. Results, subsequently reported by Gavin, Patterson, and McHenry (52), showed that the increase in liver cholesterol caused by biotin is either due to, or is coincidental with, a slight decrease in body cholesterol. It would appear that biotin causes an accumulation of cholesterol in the liver, perhaps at the expense of the amount in the body; the increase in liver cholesterol is prevented by lipocaic or by inositol. All three dietary supplements would seem to have effects upon cholesterol transport.

In a study of the influence of various B vitamins upon the production of fatty livers, Forbes (51) has secured somewhat different results. He found that the administration of nicotinic acid to rats caused an increase in liver cholesterol, in comparison to that obtained with other B vitamins but without nicotinic acid.

Unpublished observations by Gavin and McHenry on the influence of various B vitamins upon cholesterol synthesis indicate that the total amount of cholesterol approximately parallels the total amount of fat. It should be pointed out that these experiments were carried out with a high-carbohydrate, low-protein, fat-free diet, and presumably the cholesterol was synthesized, like fat, from carbohydrate. When thiamin was not supplied to the animals there was no evidence of either fat or cholesterol synthesis. These observations, and those which have previously been mentioned,

do not supply proof that any of the B vitamins are directly concerned with the formation of cholesterol. The data are consonant with the theory, expressed by many writers over a period of years, that cholesterol formation keeps pace with the amount of fat present in the body.

VII. POSSIBLE MECHANISM OF FATTY ACID SYNTHESIS AND THE RELATION OF VITAMINS TO ENZYME SYSTEMS

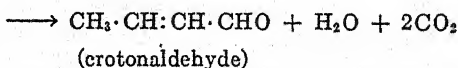
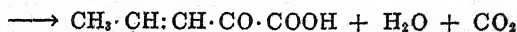
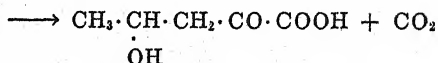
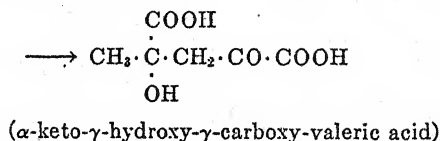
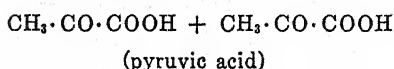
Although it was proven many years ago that animals can synthesize fats, and although this synthesis must constitute a prominent phase of metabolism, the chemical changes involved in such interconversions remain quite obscure. Several hypotheses have been advanced but in the main they have been based on either chemical information derived from *in vitro* experiments or upon biochemical observations on microorganisms. The applicability of these hypotheses to higher animals is a matter of doubt. No attempt will be made to review the theories in detail but some mention is desirable in order that a possible relationship of the B vitamins to fat synthesis may be considered.

One of the early theories regarding fat formation was that postulated by Emil Fischer (84). He believed that fatty acids could be formed from sugar molecules by direct condensation and subsequent reduction of the condensation product. For example, stearic acid would be formed from three hexose units, and palmitic acid would then be derived from stearic acid by β -oxidation. The prevalence in nature of fatty acids possessing structures which are multiples of 6 carbons favors the theory. Chemical evidence is against the acceptance of the theory, and no biochemical evidence in support of it has been forthcoming in recent years.

The importance of acetaldehyde, derived from carbohydrate, as a possible intermediate in the formation of fatty acids was emphasized by Nencki (85), by Hoppe-Seyler (85), and by Magnus-Levy (85). The theory which was developed on this basis was somewhat as follows: Units of acetaldehyde, formed by breakdown of lactic acid, underwent aldol condensation. From the aldol there were two possible products, either butyric acid or croton aldehyde which would finally be converted into butyric acid. Long chain fatty acids could be obtained by repeated condensations of aldehydes. An objection to this theory, that the condensation of aliphatic aldehydes with acetaldehyde usually yields a product with a branched-chain structure, has been found not to hold for the autocondensation of aldol (86). Aldol can be formed by mild chemical treatment of acetaldehyde (85), and the ability of the liver to effect aldol condensations has been reported (87). Evidence secured by the use of microorganisms has both supported and denied this theory. Fitz (84) was unable to demonstrate the presence of acetaldehyde or aldol in the lactate medium in which bacteria were producing butyric

acid, and Smedley-MacLean (84) reported similar observations. While studies on yeast have shown that acetaldehyde is formed during the degradation of sugar, they have failed to provide evidence as to the importance of acetaldehyde in some cases but, in others, have shown that acetaldehyde can be used in the formation of fatty acids (88, 89).

Another possible theory has been suggested by Smedley-MacLean. In this hypothesis considerable importance is given to pyruvic acid as the intermediate between carbohydrate and fat. The initial stages in the formation of fatty acids were stated by Smedley-MacLean (84) to be as follows:



It was suggested that the crotonaldehyde, which had thus been produced, might condense with another pyruvic acid molecule to yield a higher keto acid, capable of either oxidation or further condensation. This scheme would permit the formation of highly unsaturated aldehydes which could be converted into fatty acids.

Support for the hypothesis of Smedley-MacLean is furnished by several entirely different types of experimental evidence, of which the following may be cited: Unsaturated aldehydes have been identified in aerated sugar solutions in which yeast has been cultured and in which the fat content is increased (90). Yeast cells will cause the production of butyric acid and carbon dioxide from the lactone of α -keto- γ -hydroxy- γ -carboxy-valeric acid, the compound which is the first condensation product in the Smedley-MacLean sequence (84). Smythe (91) has reported that yeast cells will utilize pyruvic acid under aerobic conditions, that the respiratory quotient is increased above unity, and that the amount of ether-soluble material in the culture is increased. Smythe found that this effect could be secured with pyruvic acid in the media but that it failed to occur when acetaldehyde was substituted for the pyruvic acid.

In contrast to the results obtained by Smythe, it has been reported by

Smedley-MacLean and Hoffert (92) that yeast will bring about lipid formation from acetic acid or ethyl alcohol. Sonderhoff and Thomas (81) have suggested that fatty acids can be synthesized from the acetate ion; MacLeod and Smedley-MacLean (93) have reported observations which are also indicative of this process. Krebs and Johnson (94) have shown that animal tissues can form butyric acid from acetaldehyde. Investigations of the fates of labelled fatty acids in mice by Stetten and Schoenheimer (95) have provided information regarding fatty acid metabolism; they observed that fatty acids could undergo desaturation and also the loss of 2 carbon atoms, and that units of this size could be added to fatty acid chains. Stetten and Schoenheimer (95) suggested that the condensation by which the union would be affected would require a reactive terminal group as would be supplied by an aldehyde. While there is evidence to support the Smedley-MacLean theory, there are also data tending to confirm the previously advanced hypothesis that acetaldehyde is the likely intermediate between carbohydrate and fat. It might be suggested that pyruvic acid is the true intermediary, since acetaldehyde would likely be formed from pyruvic acid.

It is useful to consider how the theories regarding fat synthesis can be reconciled with the observations that thiamin, and perhaps other B vitamins, are essential for fat synthesis. The rôle of thiamin in this process is easier to understand than is the parts played by the other B vitamins. It is now well established that thiamin diphosphate functions as cocarboxylase in yeast (96) and, in the same form, is necessary for the enzymic disposal of pyruvic acid in more highly specialized tissues (97). Emphasis has been placed by Peters (97) upon the rôle of thiamin in the production of energy from carbohydrate. It may be that it is also needed for the production of compounds participating in the condensation reactions necessary for the synthesis of fatty acids. It is established that pyruvic acid accumulates in the tissues of thiamin-deficient animals, that a supply of the vitamin will cause a marked reduction in the amount of pyruvic acid (97), and that there is fatty acid synthesis (29). A simple explanation would be that thiamin causes the production of acetaldehyde from pyruvic acid and thus initiates the sequence of reactions which brings about the synthesis of fatty acids. This would harmonize the known information about the function of thiamin with the theory regarding acetaldehyde being the intermediate between carbohydrate and fat. It should also be pointed out that the Smedley-MacLean hypothesis requires decarboxylations, a type of reaction in which thiamin could function. It seems to be not unreasonable to suggest that the future work on the mechanism of formation of fats should include the accredited information about the function of thiamin.

While the probable rôle of thiamin in fat synthesis can be suggested, the functions of other B vitamins in this process are quite indefinite. The ex-

periments of McHenry and Gavin (38, 39, 41, 42, 43) showed that other members of the B complex, given in conjunction with thiamin will augment the amount of fat synthesis. In confirmation of this observation Quackenbush, *et al.* (47), Engel (50), and Stetten and Grail (49) have shown that pyridoxin and pantothenic acid increase the quantity of synthesized fat. While coenzyme functions have been described for riboflavin, nicotinic acid, and biotin, and suggested for pyridoxin and pantothenic acid, their rôles in fatty acid synthesis are obscure.

Discussions of the mechanism of fat synthesis have tended to assume that there is one condensation pattern by which fatty acids are built up in plants and in animals. This may be true, but there is one marked difference between fat synthesis in plants and in animals. Some plant oils contain large amounts of linoleic and linolenic acids, compounds which apparently cannot be synthesized under the conditions which have been employed in experiments on rats. It has been pointed out previously in this review that there is evidence suggesting that the character of the synthesized fat can be altered by variations in the supplements of the B vitamins. It is interesting to speculate whether differences between plant and animal fats are due, perhaps in part, to variations in the B vitamins which are present. Determinations of the amounts of the different B vitamins contained in ripening seeds might provide useful information upon which to design animal experiments for a study of the possible production of essential fatty acids.

VIII. THIAMIN-SPARING ACTION OF FATS

Derangement of carbohydrate metabolism in thiamin deficiency, manifestations of which include elevation of blood sugar, pyruvate, and lactate, led to the early recognition of the essential nature of this factor in the catabolism of carbohydrate (98, 99). The necessity for inclusion of large amounts of carbohydrate in thiamin-deficient diets in order to produce deficiency symptoms in animals (100, 101) and the capacity for fat to "spare" the vitamin has been demonstrated by several groups since the relationship was originally pointed out by Evans and Lepovsky (102, 103) in 1928-1929. These workers reported continued growth and a failure in appearance of the typical deficiency syndrome in rats when the "antineuritic-vitamin-free" diet contained 50% lard. The animals were observed for a period of six months. Subsequently, Evans and Lepovsky (104) pointed out that even on a high carbohydrate diet containing presumably an adequate amount of yeast as well as a source of the unsaturated fatty acids, animals exhibited a favorable response to the provision of additional fat. Comparison of the sparing-effects of several natural fats and oils indicated that neither the precise melting-point nor the degree of saturation influenced the activity. However, the melting-point had to be sufficiently

low to permit good absorption (105). These experiments were extended to include a study of the activities of glycerides of single fatty acids (106). Because of poor absorption, stearin was ineffective; myristin and caprylin exhibited the greatest potency. Subsequent observations reported by Salmon and Goodman (107) are in harmony with these results. They compared the thiamin-sparing capacities of various pure fatty acid esters and noted that fats having an 8-carbon structure exhibited the maximum potency. Shorter chain acids possessed toxic properties while the higher molecular weight members of the series were decreasingly active.

The sparing action of fat on thiamin requirements has been widely confirmed. Guerrant and Dutcher (108) also reported this effect, and Whipple and Church (29) described similar observations. Salmon and his associates (109, 110) have carried out comprehensive studies on this phase of the relationship of fat metabolism to vitamin activity.

In most of these investigations, the extent of change in body weight and the degree of polyneuritis were the criteria of the "thiamin state" of the animals. However, MacDonald and McHenry (111) have described the effect of fat in delaying the onset of the characteristic bradycardia of thiamin deficiency in the rat. Banerji (112) confirmed this finding and also contributed the observation that inclusion of various amounts of fat in thiamin-deficient diets proportionally counteracts the augmented excretion of bisulfite-binding substances which is typical of the syndrome.

Explanation of the mechanism of this sparing action of fat has proved to be a provocative subject. Two possible hypotheses were eliminated; Evans and Lepovsky (113) demonstrated that the activity was not attributable to any vitamin-fat complex formed in the gastro-intestinal tract, and Melnick and Field (114) showed that thiamin was absent from the fats which gave a sparing action.

Because of the typical failure in carbohydrate utilization and the associated accumulation of pyruvic acid in thiamin deficiency, the fat effect has often been interpreted as resulting from the substitution of lipid for carbohydrate as a source of energy with a consequent decrease in the need for thiamin. However, evidence indicative of fatty acid synthesis as a function of thiamin has been reported (29) and the exclusion of this observation from any explanation of the sparing effect of fat is hardly justified. For many years, deposited fat was regarded as existing in a relatively static state. By means of tracer experiments the dynamic condition of not only the phosphorus-containing lipids of kidney and liver but also of the fat depot glycerides themselves has been demonstrated. Schoenheimer and Rittenberg (20), to whom a great deal of the credit for clarification of this concept is due, have expressed the situation as follows: "The results show that the fat depots represent a much more active organ than has been thought hitherto.

... Mice, like almost all other animals, take food only at intervals; the absorbed constituents of the diet are not burned immediately but must be deposited for short periods. Part of the carbohydrate (and carbohydrate precursors) is deposited as glycogen and is always available for combustion directly in the form of carbohydrate. However, the amount of glycogen which may be stored in the organs is relatively small. Most of the absorbed carbohydrate is therefore immediately transformed into fatty acids. These are deposited in the fat depots and utilized for combustion in the postabsorptive periods." This viewpoint places a new emphasis upon the importance of fat in animal metabolism. Over a period of some years great importance has been given to carbohydrate as the prime source of energy. While carbohydrate may be the source of energy for muscular work, it is possible that fat is the fuel for basal metabolism. The body would then have a definite need for fat, a requirement which could be satisfied by the inclusion of fat in the diet or by the conversion of carbohydrate to fat under the influence of thiamin and perhaps other B vitamins. On this basis there would be a requirement for thiamin to bring about the synthesis of fat, a requirement which would be lessened by the presence of fat in the food supply.

IX. ESSENTIAL FATTY ACIDS

In 1929, McAmis, Anderson, and Mendel (115) reported the superior growth of rats provided with a small amount of fat in comparison with animals not receiving fat. In the same year, Burr and Burr (116) described a characteristic syndrome which appeared in rats fed a diet of ether-extracted casein, fat-free yeast, sucrose and salts. Under the dietary conditions described, the animals continued to increase in weight for some time but plateaued prematurely and finally, in the acute stages of the deficiency, lost weight rather rapidly. In consideration of these observations Burr and Burr discarded the explanation that the supply of a yeast factor was inadequate. They pointed out: "Whether the effect is caused by the strain of long-continued fat synthesis, suggested by Krogh and Lindhard, or whether a special type of fatty acid is required by the animal which it is unable to synthesize from the diet consumed, are unanswered questions." Relatively small amounts of lard and cod liver oil exhibited dramatic curative potencies, and the exceptional activity of liver in this regard was also indicated. Its effectiveness was superior to that of any of the fats. Subsequently Burr and Burr pointed out that the varying potencies of the fats were relatable to the structures of the component fatty acids, and the observation was made that in the animal deprived of fat the supply of an unsaturated fatty acid fraction of tissue lipids was depleted. The essential nature of dietary linoleic, linolenic, and possibly arachidonic acids was indicated (117).

Evans and Lepovsky contributed observations which are in accord with these results. They too suggested that factors other than relative inadequacy of vitamin B were concerned in the etiology of the disorders observed in animals fed strictly fat-free diets (118). The importance of the provision of unsaturated fatty acids was demonstrated by means of an experiment in which rats received a diet containing laurin as sole source of fat. Such animals exhibited even poorer growth than those receiving no fat at all (119). Sinclair, using a high elaidin diet has observed a similar relationship (120). Evans and Lepovsky also noted the depression in essential fatty acid content of the lipids of fat-starved rats (121).

The question of whether several acids were necessary or simply a key structure from which others could be formed became the subject of experimental work. Nunn and Smedley-MacLean (122) investigated the hepatic fatty acid structures of fat-deficient rats and described the absence of any acid containing 20 or 22 carbon atoms and 4 or more double bonds. Recent observations have indicated that linoleic acid, as well as being active *per se*, is used in the synthesis of arachidonic, and possibly clupanodonic acids (123, 124). Turpeinen (125) recognizing the superior curative capacity of arachidonic acid, suggested that it is of predominant importance. On the other hand, Burr, Brown, Kass, and Lundberg (126) have maintained that linoleic, linolenic, arachidonic, and cod liver oil fatty acids exhibit differences in both the qualitative and quantitative aspects of their actions. They expressed the view that, like the essential amino acids, these fatty acids should be treated as separate nutritional entities. However, in an excellent review of the subject, Burr (127) has recently pointed out that, strictly speaking, linoleic acid only is essential. It is of interest that MacKenzie, MacKenzie, and McCollum (123) reported the maintenance of rats in excellent condition for a year on a highly purified diet consisting of specially ether-extracted yeast, extracted casein, salts, and supplements of calciferol, carotene, cystine, sucrose, vitamin E, and methyl linoleate.

Burr and Burr, in an early publication (117), had suggested that the essential fatty acids were necessary for normal fat formation from non-lipid sources. Emaciated animals on the fat-free diets consumed as much food as the normal controls but apparently oxidized it, since growth and fat synthesis were not evident. Smedley-MacLean and Nunn (129) failed to confirm these observations and indicated that, although the capacity of the animal to store fat was impaired, the fatty-acid-synthesizing mechanism remained intact. Subsequently, however, these authors rejected this hypothesis and concluded that the polyunsaturated acids were concerned with neither the synthesis of fat nor its storage. Rats existing on the fat-deficient diet for long periods, contained actually a higher proportion of

lipids than controls which had received linseed oil. During the first six months on the fat-deficient diet the rats' supply of polyunsaturated fatty acid was reduced to a low level. During the second six month period, growth having ceased, the rate of utilization of these substances was markedly depressed. On the basis of observations such as these, the suggestion was advanced by Smedley-MacLean, *et al.* that these acids function in some as yet unelucidated way, in the formation of new cells. The growth of the Walker tumour in normal rats was associated with a decrease in the amount of subcutaneous fat and a depression in the proportion of highly unsaturated fatty acids (130). Whatever may be the functions of unsaturated fatty acids in the growth process they are probably involved also in fat transport. Engel (50) has shown that the lipotropic action of choline is evident only when linoleic acid is provided. Thus, both these phospholipid constituents must be available.

The limited information available on fatty acid deficiency in species other than the rat has been reviewed by Burr (127). Attention should be drawn to an interesting observation made on an adult human who existed for a six month period on a fat-free diet (132). The arachidonic and linoleic acid contents of the serum lipids were depressed and, in the post absorptive periods, active fat synthesis was indicated by respiratory quotients well above unity.

The essential fatty acid problem has been rendered more complex by experiments which indicated a possible relationship to the metabolism of the B vitamins. Attention having been drawn to the similarity between the acrodynia of pyridoxin deficiency and the dermal abnormalities of unsaturated fatty acid deficiency (133), observations published by Birch and György (134) suggested that fat exerted a sparing action on the metabolism of pyridoxin. Quackenbush, *et al.* (135) reported that the symptoms developed on a diet lacking pyridoxin could be alleviated by the administration of various natural fats and synthetic esters and the activity of the fats in this regard paralleled their degree of unsaturation. Linoleic acid was apparently capable of curing both the acrodynia and the essential fatty acid syndrome. The activity of the fat could not be attributed to pyridoxin contained in it. It was pointed out that the acrodynia could also be relieved by means of a rice bran concentrate (136).

Results published by Salmon, however, were not in harmony with these observations. He reported that both a heated yeast extract and certain oils were necessary for the maintenance of a normal skin condition (137), and Birch (138) also indicated that both fat and water-soluble factors were necessary. Subsequently Salmon showed that the dermatitis in rats provided with casein, sucrose, salts, carotene, calciferol, α -tocopherol, thiamin,

riboflavin, and choline could be cured completely only by the administration of pyridoxin, linoleic acid, and pantothenic acid (139).

Recently Quackenbush, Steenbock, Kummerow, and Platz (140) reviewed the situation and pointed out that the discrepancies among the results previously reported could be attributed to the use of diets of questionable lipid content and undefined sources of water-soluble factors. In rats given a highly purified, fat-free diet, curative tests showed that linoleic acid was the primary factor concerned. In sufficient amounts it was completely effective; in subcurative amounts pyridoxin enhanced the action of the fatty acid. Prophylactic tests indicated that pyridoxin was capable of retarding the development of the skin condition, and pyridoxin plus pantothenic acid were still more effective. In this publication it was pointed out that the "accessory factor" of rice bran concentrate reported by Schneider, *et al.* (136) to be necessary in addition to pyridoxin in the cure of acrodynia appeared to consist of pantothenic acid as well as some other substance. As stated by the authors, "whether the additional factor is a known dietary essential remains to be determined." Although a relationship of these several factors to the metabolism of certain fatty acids has been indicated there has been no suggestion as to the mechanism of action, nor, indeed, proof that such a relationship exists.

It seems odd that an organism possessing mechanisms which make possible the synthesis of structures such as oleic, stearic, and palmitic acids and capable of desaturating, hydrogenating, and adding carbon atoms to a fatty acid chain would be incapable of promoting such closely allied reactions as the formation of linoleic, arachidonic, and other acids. In view of the observation of Sinclair (120) that rats exhibited superior growth on a high carbohydrate, fat-free diet in comparison with those on a high-elaidin regimen, it is interesting to consider the two possible explanations presented by the author. He pointed out that either the ingestion of fat augmented the *requirement* for certain fatty acids, or that the inclusion of fat in the diet suppressed the process of *synthesis* of fat, including that of the unsaturated acids essential for growth.

The possibility thus remains that the animal body may be capable of synthesis of these acids when the necessary dietary constituents are provided. In much of the work outlined, yeast was used as the source of the B vitamins. Some attention has been drawn to the inadequacy of the diets employed in this respect, and an increase of the yeast intake has been tested for effects upon the syndrome (32, 33). It was usually concluded that the disorder could not be related to adequacy of any B factor. However, according to R. J. Williams (141) the vitamin content of brewers' yeast assayed as follows, by microbiological methods:

Factor	γ per g.
Thiamin.....	8.5
Riboflavin.....	15.2
Nicotinic acid.....	126.0
Pantothenic acid.....	42.5
Pyridoxin.....	1.0
Biotin.....	0.071
Inositol.....	280.0
Folic acid.....	1.05

Since rats in experiments on unsaturated fatty acids usually received somewhat less than 1 g. of yeast per day the B vitamin adequacy of the diets may have been borderline in some respects and, as a source of biotin, quite sub-optimal. In this regard Burr's observation on the superior efficacy of liver should be recalled (116) as well as the curative properties of a rice bran concentrate (136). Further, MacKay and Barnes (142) contributed the interesting observation that the dermal disorders manifest in rats which were fed 10% egg white could be completely cured by the replacement of some of the dietary crisco with corn oil in addition to the provision of extra pyridoxin. The diet used contained 5% yeast, a quantity which would provide suboptimal amounts of pyridoxin. The possibility of the synthesis of essential fatty acids in long-term experiments in which optimal amounts of all of the B vitamins are provided should be the subject of further investigation.

X. SUMMARY

In recent years experimental data have shown that several of the B vitamins are necessary for the *in vivo* synthesis of fats from carbohydrates or protein. This is not surprising since thiamin, riboflavin, and nicotinic acid have been proven to be constituents of enzyme systems which are essential for metabolism. It is entirely likely that pyridoxin, pantothenic acid, and biotin act in a similar capacity. While little is definitely known regarding the chain of reactions by which fat is formed from protein it has been assumed for some years that the formation of fat from carbohydrate begins after the degradation of carbohydrate has proceeded to the pyruvic acid stage; the carboxylase system, containing thiamin, is necessary for the utilization of pyruvic acid. To regard the effects of the B vitamins upon fat synthesis as fortuitous adjuncts of "growth" produced by these vitamins is an attitude which overlooks what is already known about the fundamental enzymatic activities of the B vitamins and which might retard an advancement in knowledge of metabolism. Most of the B vitamins are now available as pure chemical substances. What is now needed is not continued emphasis upon "growth", or skin lesions, or other superficial phenomena, but research upon the fundamental biochemical reactions in which these

vitamins serve, and derangement of which are responsible for the superficial lesions. The splendid success of attempts to isolate and synthesize various vitamins has opened new pathways and provided more exact methods for the study of metabolism. The sequence of events in the intermediary metabolism of carbohydrates, proteins, and fats has long remained obscure. New opportunities are available for the study of metabolism; old methods and concepts may suffer. If many of the vitamins, acting as integral parts of enzyme systems, are necessary for metabolic reactions, these vitamins should be considered in studies on metabolism. In future investigations on fat synthesis and metabolism it will no longer be sufficient to state that the animals were fed "an adequate diet"; careful consideration should be given to the B vitamin supplements, both qualitatively and quantitatively.

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The Chemistry of Biotin

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INTRODUCTION

With the demonstration early in 1940 of the importance of biotin in animal nutrition, much interest has been aroused in this new member of the vitamin B complex. Intensive chemical work directed toward the determination of the structure of biotin has been in progress since 1936 in Europe and during the past three years in this country. Several papers have appeared since 1940 on various phases of the problem of the structure of this vitamin. Recently evidence for the complete structure of the biotin molecule has appeared, and so it seems appropriate at this time to gather the chemical data which has been accumulated into a single review. It is the purpose of this paper, therefore, to present the complete story of the chemical investigations on biotin which have appeared in the literature, together with a short review of the history of the vitamin and an indication of its importance in the field of biochemistry.

I. HISTORICAL

Since the year 1901, when a publication by Wildiers (110) first described the stimulating effect of small amounts of organic material on the growth of yeast, this effect has been the subject of many publications, and only after several years did the original observations become universally accepted. The name "bios" was given by Wildiers to the substance or substances causing the increased growth of yeast. In later years bios was shown to be multiple in nature, and was fractionated into Bios I, precipitated by basic lead acetate; Bios IIa, unadsorbed on charcoal; Bios IIb, adsorbed on charcoal; etc. In 1928 Eastcott (25) identified Bios I as *meso*-inositol, and in 1930 Williams and Roehm (113) demonstrated that thiamine (probably Bios V) was a yeast-growth factor. Further work has shown that pantothenic acid (112) (which appears to be Bios IIa), β -alanine (114), and pyridoxin (94) all exert definite stimulatory actions on yeast growth.

The Bios IIb, or charcoal-adsorbable fraction attracted the attention of Kögl, and in 1935 he announced (54) the isolation from egg yolk of minute amounts of a crystalline compound possessing the greatest part of the yeast-growth activity of the Bios IIb fraction. This compound Kögl called "biotin." In 1936, Kögl and Tönnis (60) presented in detail a description of their isolation procedure, and described some of the chemical and physical characteristics of the crystalline material, although characterization of the compound was not complete because of the small amount of material isolated. During the next two years Kögl (55, 56) stated that the crystals he had obtained were the methyl ester of the active compound, biotin, and possessed the empirical formula $C_{11}H_{19}O_5N_2S$.

In 1933, Allison, Hoover, and Burk (1) described the growth- and respiration-promoting effects of extracts from various sources for *Rhizobium trifolii*, a legume nodule organism. The active agent was named "coenzyme R." In 1939, West and Wilson (107) pointed out the similarity between the effects of coenzyme R and biotin concentrates on the growth of yeast and *Rhizobium trifolii*, and Nilsson, Bjälfve, and Burström (78) found that a sample of Kögl's crystalline biotin possessed coenzyme R activity. It appeared, therefore, that coenzyme R was identical with biotin.

It will be worthwhile at this point to leave for the moment the discussion of biotin, the yeast-growth factor, and turn to an apparently unrelated investigation in the field of mammalian nutrition.

Boas described, in 1927, the effects produced in rats when large amounts of dried egg white were added to the diet (4). On such a regime the animals gradually lost their hair; dermatitis, skin hemorrhages, and loss of body weight occurred; a spasticity developed, and death ultimately resulted.

Boas also found that there occurred in a variety of foodstuffs a "protective factor X," which would cure and prevent the syndrome produced in rats by an egg white diet. Parsons and her associates studied extensively the egg white injury factor and the distribution of the protective factor, and found that the toxic factor in egg white was apparently protein in nature (82), and that the toxicity syndrome could be produced in rats, chicks, rabbits, and monkeys (68). Injectable extracts of the protective factor were prepared (66), but it remained for György and his coworkers to investigate the chemical and physical properties of the protective factor, which he called "vitamin H" (from the German *Haut*, skin).

György (32) described a diet used to produce the deficiency syndrome in rats for assay purposes, and investigated quantitatively the distribution of vitamin H in foodstuffs. György, Kuhn, and Lederer (33) described the liberation of vitamin H in a water-soluble, dialyzable form from liver and yeast, in which it occurs chiefly as a high-molecular weight complex. Partial concentration of fractions from liver was accomplished. Birch and György (3) showed that vitamin H possessed an isoelectric point in the neighborhood of pH 3.0 to 3.5.

Early in 1940, György, Melville, Burk, and du Vigneaud (34) suggested the possible identity of vitamin H with biotin and coenzyme R, based on the biotin, coenzyme R, and vitamin H activities of electrodialysis fractions of a vitamin H concentrate from liver. A short time later du Vigneaud, Melville, György, and Rose (20) established the identity by vitamin H assays of a sample of crystalline biotin supplied by Professor Kögl. Final confirmation was afforded by the demonstration by György, Rose, Hofmann, Melville, and du Vigneaud (39) of the high vitamin H activity of pure crystalline biotin methyl ester isolated from liver concentrates high in vitamin H activity.

II. METHODS OF DETERMINATION

Up to the present time no chemical method has been devised for the determination of biotin. Several methods based on the acceleration of growth of microorganisms by biotin have been described. Kögl and Tönnis (60) utilized the growth of Rasse-M-yeast over a 5-hour test period as an assay method in their isolation of biotin. At Cornell University Medical College the method of assay described by Snell, Eakin, and Williams (98), in which the growth of a small seeding of *Saccharomyces cerevisiae* after a 20-hour incubation period is measured, was found highly satisfactory in developing the procedure used for the isolation of pure crystalline biotin from liver (17) and milk (73) concentrates. The great physiological potency of biotin becomes apparent in these tests; a readily-observable increase in yeast growth is brought about by a concentration of

less than 1 part of biotin in 5×10^{11} parts of the medium. Other microorganisms which have been used for the assay of biotin include *Clostridium butylicum* (86), *Rhizobium trifolii* (108), *Staphylococcus aureus* (88), *Ashbya gossypii* (93), *Lactobacillus arabinosus* (99), and *Lactobacillus casei* (97). In the case of yeast the methyl ester of biotin shows the same activity per mole as the free acid; certain other organisms, however, appear to be unable to utilize the ester for growth purposes, and ester preparations must be hydrolyzed before being assayed.

Snell, Eakin, and Williams (98) showed the necessity for liberating biotin from the high-molecular weight complex in which it chiefly occurs, before samples are used in the yeast-growth method of assay. Liberation processes utilize autolysis, acid hydrolysis, or enzymatic digestion (62, 102, 103, 104).

Microbiological assay methods for biotin have supplanted for most purposes the slow and laborious rat assay method originally used in investigations of vitamin H (32). Large numbers of rats are necessary to obtain reasonably accurate quantitative data, and six to eight weeks are required for completion of the tests. The vitamin H unit has been defined as the daily dose of material which will bring about complete cure of the egg white injury in the rat in 4 weeks. Pure biotin has been shown to possess a vitamin H activity of approximately 28,000 units per mg., i.e., the daily curative dose is approximately 0.04γ (39, 18). Parenterally-administered biotin appears to be three to five times as effective as the orally-administered compound (36).

The chick has been suggested (2) for use in assays of biotin. The biotin requirement of chicks is higher than that of rats, and the deficiency syndrome can be brought about in chicks by a heated or purified diet which does not contain egg white (2, 45).

III. OCCURRENCE

By means of the extremely sensitive microbiological assay methods, biotin has been found to be widely distributed and indeed appears to be a common constituent of every type of living cell. In keeping with its high physiological activity, biotin is present in very low concentrations in living tissues. One of the richest sources, liver, contains less than 0.0001 per cent of biotin. Such low concentrations of biotin make necessary the use of extremely large amounts of starting material for isolation procedures, and make the isolation of large amounts of the crystalline material difficult and almost prohibitive.

The distribution of biotin has been studied by several workers. György (32) investigated the vitamin H activity of several foodstuffs by the less-sensitive rat assay method. Kögl and von Hasselt (61) studied the biotin

content of various animal tissues, although at that time the importance of bound biotin was not understood. Kögl and Haagen-Smit (58) described the biotin content of several plant seeds, and Lampen, Bahler, and Peterson have listed biotin values for various vegetables, fruits, nuts, and animal products (62). Williams and his associates have made extensive investigations of the vitamin contents (including biotin) of many tissues and foodstuffs (103, 104). The richest sources of biotin are liver, kidney, yeast, pancreas, and milk. The biotin content of tumors has been investigated by West and Woglom (109) and by Williams and co-workers (104).

IV. BIOCHEMICAL SIGNIFICANCE

Biotin has been found to be an essential factor in the biochemistry of many diversified types of living organisms. The demonstration in 1940 of the vitamin properties of biotin has stimulated a large amount of research during the past few years on the physiological action of this vitamin, and a wealth of information is being collected. Much remains to be done, however, particularly in the elucidation of the mechanism of action of biotin *in vivo*.

1. Plants and Microorganisms

Biotin enjoys a wide distribution in the vegetable kingdom. It is present in relatively high concentrations in the seeds of plants, and increases in amount during the germination of many kinds of seeds (10). The vitamin has been shown to function as a growth hormone for isolated pea roots (61).

In the field of microorganisms biotin has been found to be an essential growth substance for numerous strains of yeast, bacteria, and fungi. Of the microorganisms tested, those which do not require added biotin for growth have been found to possess the power to synthesize biotin (63, 64). It seems likely, therefore, that biotin is an essential nutriment for all types of microorganisms, and that the need for an exogenous supply of the vitamin is governed only by the ease with which biotin can be synthesized by the organism. The mechanism of action of biotin on microorganisms appears to be not necessarily a direct effect on growth, for biotin may markedly increase the respiration of *Rhizobium* and the fermentation and respiration of yeast without concomitant growth (1, 9).

2. Animal Nutrition

Biotin (vitamin H) is a necessary dietary constituent for almost all types of animals so far studied; these include the rat, dog, rabbit, monkey, chick, turkey, and man. With the exception of the chick and turkey, the presence

of egg white in the diet is necessary to cause the appearance of deficiency symptoms, possibly because of the lower biotin requirements of these species. In addition to the difficulty in obtaining a completely biotin-free diet, the synthesis of biotin by intestinal flora complicates the problem. Recent work directed toward the elimination of such a microbial synthesis of vitamins has utilized the inclusion in the diet of the relatively non-absorbable sulfa drugs. In such experiments in which rats were fed purified diets containing succinyl-sulfathiazole or sulfanilyl-guanidine, it was found that the dermatoses which developed without the inclusion of egg white in the diet could be cured or prevented by biotin administration and that biotin promoted growth (13, 70, 76).

a) *Avidin*. The pathological effects produced by dietary egg white result from an induced biotin deficiency due to the formation of a non-absorbable complex between the biotin in the digestive tract and a constituent of the egg white, for the tissues of animals on a diet containing egg white and adequate amounts of biotin possess significantly lowered biotin concentrations, while the feces contain large amounts of the bound vitamin (23, 81). The substance present in egg white which produces the toxic effects (38) has been isolated in crystalline form (85). This substance, which has been named "avidin" because of its avidity for biotin, is a basic protein with a molecular weight in the neighborhood of 70,000, and appears to combine with biotin in a stoichiometric ratio of one molecule of biotin for one molecule of avidin (115). The combination of biotin with avidin occurs *in vitro* (24); biotin is not released from the combination by dialysis over a wide range, but is completely released by steam sterilization (37) and can be released by ultraviolet irradiation (40). Proteins associated with eggs appear to be the only types which show this marked biotin-combining effect (46, 106, 115). Of interest is the finding that the avidin-biotin combination when administered parenterally appears to be split in the body to liberate utilizable biotin (37).

A clue as to the nature of the linkage between biotin and avidin has been suggested by du Vigneaud, Dittmer, Hofmann, and Melville (15), who showed that the diaminocarboxylic acid derived from biotin by the hydrolysis of the urea group does not combine with avidin. This indication that the presence of a urea configuration in biotin is essential for the combination with avidin has also been borne out by comparative studies of the avidin-combining properties of other derivatives of biotin (unpublished data).

b) *Biotin in Rat Nutrition*. Several workers have described the egg-white injury syndrome in the rat (4, 80, 95, 96, 100). György (32) pointed out the difference between the egg white injury syndrome and the skin manifestations exhibited in pellagra and acrodynia. Briefly, the symptoms

are a generalized, pruritic, exfoliative dermatitis, coupled with seborrhea, alopecia, poor growth, and an abnormal "kangaroo-like" posture. Administration of biotin produces a rapid return to normal gait and posture, the dermatitis disappears, and a new growth of hair covers the denuded areas. The typical denudation around the eyes, first noticed by Boas in biotin-deficient rats, and curable by the administration of biotin, has been identified with the "spectacle eye" condition studied by other workers (75). Biotin deficiencies may also bring about a change in the color of the fur of rats (26, 35, 80).

It has been suggested (117) that the achromotrichial action of biotin and folic acid may be due primarily to their effect on pantothenic acid metabolism.

The mode of action of biotin in animal tissues has been suggested to be that of a component of an enzyme system concerned with the metabolism of pyruvate, since rats on a pantothenic-acid- and biotin-deficient diet showed decreased rates of oxidation of pyruvate by the liver (87).

c) *Biotin in Avian Nutrition.* The importance of biotin in chick nutrition has been recognized for several years (67). Deficiency symptoms can be produced in the chick and in the turkey by a low-biotin or heated ration without the necessity of adding egg white to the diet (2, 44, 83). Biotin appears to be necessary for the normal embryonic development of hen eggs (12). Varying results, depending on the type of diet, have been obtained for the effectiveness of biotin in preventing perosis (52, 91).

d) *Biotin in Human Nutrition.* One of the first attempts to link biotin (vitamin H) deficiency with pathological symptoms in the human was the observation of Findlay and Stern (29) in 1929 that some of the symptoms exhibited by biotin-deficient rats possessed a similarity to those shown by children with Swift's disease or "pink disease." György (31) has noted the close similarity between certain aspects of the syndrome in animals and the symptoms of seborrheic dermatitis in man. The first indication of the need for biotin by man was described during the year 1942 (101). Four humans were placed on a controlled diet containing sufficient desiccated egg white to provide 30 per cent of the caloric intake, and supplemented with minerals and the known essential vitamins. On this diet the subjects developed a desquamative dermatitis and pronounced pallor of the skin and mucous membranes. These symptoms were accompanied by mental depression and lassitude, and muscular pains. The biotin excretion in the urine diminished to almost a tenth of its normal value. The pathological conditions were rapidly cured by the administration of a concentrate equivalent to 150-300 γ of biotin daily. It is unfortunate that pure biotin was not used, so that unequivocal conclusions as to the need of the human for biotin could be drawn.

Recently a clinical case of a skin disorder has been reported which appeared to be due in part to a biotin deficiency resulting from a restricted diet containing a large proportion of raw eggs (111).

e) *Biotin and Neoplasms*. The biotin content of tumors was shown by Kögl and von Hasselt (61) to be higher than that of normal tissues. In 1941, in studies on the effect of protective agents against the formation of liver tumors in rats due to dietary *p*-dimethylaminoazobenzene ("butter yellow"), it was discovered that a greater incidence of tumors occurred when biotin was administered (21). Embryonic tissue, which possesses in common with tumors the property of rapid growth, also contains comparatively large amounts of biotin; in general the biotin level of tumors deviates from the normal adult values in the same direction as that of corresponding embryo tissues (109). The attractive hypothesis that a reduction in the incidence or a regression of tumors might be accomplished by limitation of the biotin supply has been put to the experimental test by feeding avidin or egg white, but with little success. In rats, dietary avidin depleted both normal and tumor tissues of biotin, but the tumors studied were capable of maintenance in the relative absence of biotin. Rhoads and Abels (90) have recently reported negative results in a clinical study of the feeding of low-biotin diets containing egg white and avidin to two patients with cancer.

f) *Biotin and Fat Metabolism*. Gavin and McHenry (30) have obtained fatty infiltration of the livers and an increase in the body fat of rats by the administration of biotin. The fatty livers were characterized by a high cholesterol content, and resembled those previously produced by feeding a beef liver fraction (69). The effect of biotin was prevented by simultaneously feeding egg white, lipocaic, or inositol, but choline was ineffective. Of interest in this connection is the original observation of Boas (4) that rats which had suffered severe biotin deficiencies possessed almost no stores of body fat.

g) *Specificity of Biotin*. No other well-defined, biologically-active and chemically-unrelated compounds have been found to possess the special physiological activities ascribed to biotin. Few compounds structurally related to biotin have yet been tested biologically; most of those so far tested have been products and derivatives obtained during the degradative study of the structure of biotin.

It appears that all the functional groups of biotin (carboxylic acid, urea, and thio ether) are highly specific for full biotin activity. Thus, the diaminocarboxylic acid derived from biotin by hydrolysis of the urea ring shows approximately 10 per cent of the activity of biotin itself in supporting the growth of yeast, at concentrations which bring about one-half the maximum growth, and does not combine with avidin (15). The

availability of the methyl ester of biotin for yeast growth is undoubtedly due to the ability of yeast to hydrolyze the ester, inasmuch as the ester is unable to support the growth of certain other organisms which utilize the free acid (97). Likewise, the sulfone of biotin possesses approximately one-thousandth the yeast-growth promoting power of biotin. The sulfoxide of biotin, however, appears to possess approximately the same activity as biotin for yeast growth, probably by virtue of its reduction by yeast to biotin. The sulfone, on the other hand, would not be expected to be reduced. The recently-observed inactivation of biotin by dilute H_2O_2 (77) and by rancid fat (84), whereby the biotin is rendered non-utilizable for *Lactobacillus casei* but still will support the growth of yeast, is undoubtedly due to the formation of biotin sulfoxide.

Pimelic acid has been shown to be an essential nutrilit for the diphtheria bacillus. Although the growth of this organism in the presence of pimelic acid is not affected by biotin, it has been shown by du Vigneaud, Dittmer, Hague, and Long (14) that in the absence of pimelic acid the diphtheria bacillus does require biotin for growth. Further indication that pimelic acid may be a precursor in the microbiological synthesis of biotin was offered by Eakin and Eakin (22) who demonstrated an increased synthesis of biotin when pimelic acid or pimelic acid and cystine were added to *Aspergillus niger*, an organism which requires neither biotin nor pimelic acid for growth. Some organisms, however, apparently cannot utilize pimelic acid in the synthesis of biotin (92, 116).

Oppel (79) has recently shown that a considerable proportion of the biotin activity of various urines, as measured by the yeast-growth method, cannot be inhibited by treatment with avidin. This demonstration of the existence of a naturally-occurring, avidin-uncombinable substance possessing biotin activity has been further investigated by Burk and Winzler (8). They found that the yeast-growth activity of the avidin-uncombinable fraction was greatly reduced by boiling or autoclaving at physiological pH values. This avidin-uncombinable fraction was found to be widely distributed in tissues and foodstuffs and was synthesized by bacteria. An avidin-combinable fraction inactive for yeast-growth but active for the growth of *Rhizobium* was also discovered in urine. These workers postulated that biotin may act physiologically as a coenzyme of CO_2 transfer, by virtue of an opening and closing of the urea ring of biotin.

V. ISOLATION

The isolation of crystalline biotin methyl ester by Kögl and Tönnis (60) from dried Chinese duck egg yolk was accomplished by some sixteen separate steps, including extractions with hot and cold water, precipitation procedures with acetone, alcohol, lead acetate, phosphotungstic acid,

mercuric chloride, and bromopicolonic acid, and charcoal adsorptions. The final steps of the isolation were esterification of the concentrates with methanol-HCl, high-vacuum distillation of the esterified material, and crystallization of the distilled biotin methyl ester from a chloroform-petroleum-ether mixture. In this way from 250 kg. of dried egg yolk Kögl and Tönnis obtained 1.1 mg. (1.8 per cent of the biotin present in the starting material) of crystals of biotin methyl ester with a melting point of 148°. In 1941, Kögl and Pons (59) described modifications of the isolation procedure which raised the yield of crystalline ester up to 10 to 20 per cent. The major changes were the use of Chinese dried hen egg yolk in place of duck egg yolk, distillation of the high-potency oils in a small molecular still, and the use of mesityl oxide as the crystallization-solvent for the biotin methyl ester. The biotin methyl ester obtained in this way possessed a melting point of 153° (uncorrected). Further crystallization from methanol-ether raised the melting point to 161.5°.

Rainbow and Bishop (89) in 1939 described an isolation procedure with which they had succeeded in effecting over a million-fold concentration of biotin from egg yolk. Their highly active fraction was not purified completely, however.

György, Kuhn, and Lederer (33) in 1939 described the preparation of vitamin H concentrates from liver, with a concentration of the active principle of 2500 to 3500 times. In 1940, du Vigneaud, Melville, György, and Rose (20) showed definitely that biotin possessed vitamin H activity. In order to make certain that the active agent of the vitamin H concentrates which were being studied at Cornell Medical College was identical with the crystalline compound obtained by Kögl from egg yolk, the isolation of the agent was undertaken. Later in the same year György, Rose, Hofmann, Melville, and du Vigneaud (39) described crystalline biotin methyl ester isolated from the vitamin H liver concentrates, and in a later paper du Vigneaud, Hofmann, Melville, and György (17) published details of the isolation procedure.

The successful procedure leading to the crystalline compound was one based on chromatographic adsorption techniques. The vitamin H liver concentrates used as the starting material were supplied by Dr. György and had been prepared from the alcohol-insoluble fraction of beef liver by high pressure hydrolysis, precipitation of inert material with alcohol and acetone, and precipitation of the active substance with phosphotungstic acid, followed by decomposition of the precipitate with barium hydroxide (33). This crude material, containing approximately 0.1 per cent biotin, was subjected to esterification conditions with methanol and HCl, and the ethyl-acetate-soluble material from this procedure was dissolved in chloroform and passed through a column of aluminum oxide (standardized

according to Brockmann). The column was washed with chloroform, and the active material was eluted with a 10 per cent methanol-acetone mixture. By this procedure a forty-fold concentration of the active principle was accomplished; an additional three-fold concentration was achieved by a second adsorption on aluminum oxide and elution. The active material from this second elution was dissolved in chloroform, and the solution was extracted with dilute hydrochloric acid. The acid extract contained most of the active material; this was re-esterified, and the ester was extracted from the esterification mixture with ethyl acetate. Concentration of the ethyl acetate solution resulted in the separation of crystals of the methyl ester of biotin. The crystals were purified by two crystallizations from methanol-ether mixtures, and then by sublimation in a high vacuum (10^{-5} mm.). The sublimate was crystallized from methanol and ether. In this way there was obtained a total of 70 mg. of long fine needles, m.p. $166-167^{\circ}$ (uncorrected). The yield of pure material was 38 per cent, based on the vitamin H concentrate used.

The biotin methyl ester so prepared was soluble in methanol, ethanol, acetone, and chloroform, sparingly soluble in ethyl acetate, and almost insoluble in water and ether. The compound was optically active, $[\alpha]_D^{22} = +57^{\circ}$ for a 1 per cent solution in chloroform. Kögl and Pons (59) found biotin methyl ester to possess a rotation of $[\alpha]_D^{15} = +82^{\circ}$ in methanol.

The preparation of the free acid from the methyl ester of biotin was described by du Vigneaud, Hofmann, Melville, and Rachele (18). The methyl ester was readily saponified with dilute alkali at room temperature; acidification of the saponification mixture with hydrochloric acid and concentration of the acidified solution yielded crystalline biotin, which was recrystallized from water. The pure biotin crystallized in long, fine needles, as shown in Plate I, and melted with some decomposition at $230-232^{\circ}$ (uncorrected). It was soluble in dilute alkali and hot water, sparingly soluble in dilute acid and cold water, and practically insoluble in organic solvents. A 0.3 per cent solution of the compound in 0.1 *N* NaOH showed an optical activity of $[\alpha]_D^{22} = +92^{\circ}$.

Melville, Hofmann, Hague, and du Vigneaud (73) later described the isolation of biotin from a more readily available source. This starting material was a milk concentrate containing 0.1 to 0.2 per cent biotin. One gram of biotin in the form of this concentrate represented approximately 34,000 lbs. of milk. The procedure was a modification of that used for the isolation from liver. The biotin in the crude concentrates was converted to the methyl ester, and the ester was adsorbed on Decalco from chloroform solution and eluted with a 5 per cent methanol-acetone mixture. This preliminary adsorption step furnished a 5-fold increase in potency of the concentrate; adsorption of this material on a column of

commercial activated alumina (Alorco) and elution with a 10 per cent methanol-acetone mixture yielded fractions containing 10 to 20 per cent biotin. Removal of solvent from these fractions yielded the crystalline methyl ester from which the non-crystalline material present was removed almost completely by washing with ethyl acetate. The biotin methyl ester was further purified by sublimation *in vacuo* and crystallization from a methanol-ether mixture. (It was necessary to use peroxide-free ether to prevent destruction of the biotin.) The pure ester, m.p. 166-167°, was saponified with dilute alkali, and the free acid was recrystallized from water. The over-all yield of biotin, m.p. 230-232°, based on the milk concentrate used was 25 to 40 per cent.

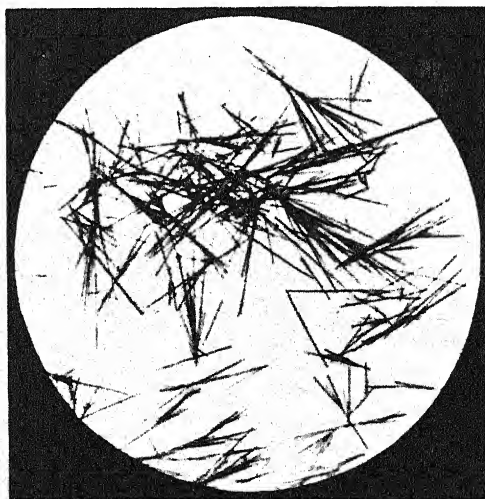


Plate I
Biotin (Free Acid) Crystallized from Water
Magnification 100 X

The biotin methyl ester obtained from vitamin H liver concentrates and from milk agreed in all its properties with those described by Kögl for the ester isolated from egg yolk. The corrected melting point of the ester was in agreement with that given by Kögl for his purest material (59). This agreement in properties, together with data subsequently obtained on derivatives and degradation products of biotin left little room for doubt that both groups of workers were dealing with one and the same compound.

VI. CHEMISTRY

Up to the year 1941, knowledge of the chemical structure of biotin was limited to two observations mentioned by Kögl (56) in a 1938 address:

the empirical formula for biotin methyl ester was $C_{11}H_{18}O_3N_2S$, and this compound required 1 equivalent of alkali for saponification. Earlier work had necessarily been limited to inactivation and re-activation experiments on biotin concentrates, in which a change in the physiological activity of the concentrate served as a criterion for a change in the biotin molecule. Obviously such experiments, valuable as they are, yield results which of necessity are limited in scope and which must be interpreted with caution. It is not surprising, therefore, that fundamental advances in the knowledge of the chemistry of biotin had not been made sooner. The tedious and expensive methods required for the isolation of biotin, coupled with the extremely small amounts of the pure compound which have been available for study, have served to delay progress in the chemical investigation of biotin even with improved methods of isolation.

Kögl and Tönnis (60) in 1936 stated that biotin formed an inactive acetyl derivative; with benzoyl chloride in pyridine, activity was also lost. At that time Kögl and Tönnis reported biotin to contain nitrogen but no phosphorus or sulfur, and it was not known definitely whether the methanol-HCl treatment of biotin formed an ester, a lactone, or a lactam. Catalytic hydrogenation had no effect on the activity of the crystalline compound. A diffusion experiment indicated a molecular weight in the neighborhood of 200. György, Kuhn, and Lederer (33) likewise found that biotin (vitamin H) was inactivated by benzoyl chloride in pyridine, and in addition stated that formaldehyde, nitrous acid, ketene, and hydrogen peroxide all led to inactivation. In 1940, Snell, Eakin, and Williams (98) demonstrated that the inactivation of biotin by nitrous acid occurred at a rate commensurate with the rate of destruction of an α -amino acid, in contradistinction to a β - or γ -amino acid. From the foregoing work, therefore, it appeared that biotin contained both an acidic and a basic group, and possibly was an α -amino acid, albeit a predominantly acidic one (3). From the work of Brown and du Vigneaud (6), however, in which a series of inactivation experiments on very small amounts of crystalline biotin was carried out, it was demonstrated that ninhydrin had no effect on biotin activity (as Rainbow and Bishop (89) had found earlier with a biotin concentrate), thus casting doubt on an α -amino acid configuration. It was likewise shown that acylating, alkylating, and reducing agents, under the conditions employed, did not destroy biotin activity. The activity was destroyed, however, by oxidizing agents and by prolonged treatment at elevated temperatures with strong acid or alkali.

When crystalline biotin was isolated from liver (17) and milk (73) the way was opened for a direct chemical attack on the structure of the compound.

1. The Presence of a Carboxyl Group in Biotin

Analyses of the crystalline compound isolated from esterified liver concentrates at Cornell Medical College led to the empirical formula $C_{11}H_{18}O_3N_2S$, which is identical with the formula given by Kōgl (56). Saponification of this compound with alkali and acidification of the saponification solution yielded crystals which when analyzed gave the empirical formula $C_{10}H_{16}O_3N_2S$ (18). The loss of one methylene group by the alkaline treatment indicated the saponification of a mono-methyl ester. Re-esterification of the free acid could be accomplished by the use of diazomethane; the methyl ester produced in this manner was identical

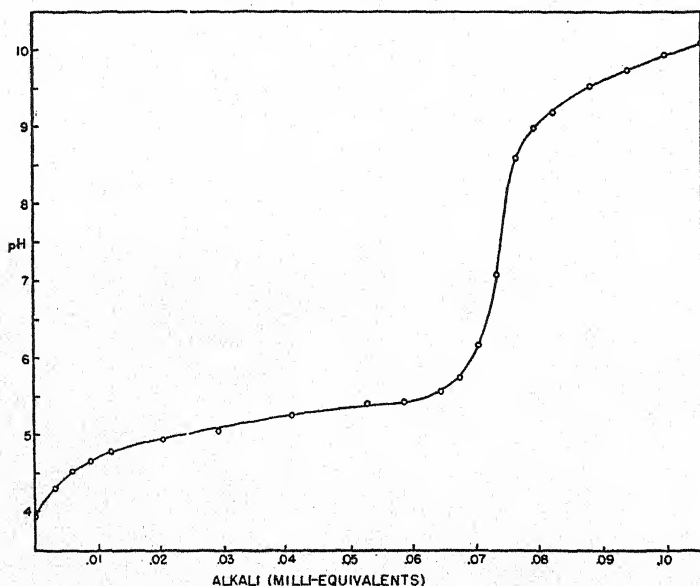


Fig. 1. Electrometric Titration of 17.99 mg. of Biotin with NaOH

with that isolated from esterified liver fractions. An electrometric titration of the free acid with NaOH was carried out with a Beckmann pH meter. The curve obtained (Fig. 1) was quite similar to that expected from a simple monocarboxylic acid, and the value of 244 for the neutral equivalent obtained from the inflection point of the curve agreed with the theoretical value calculated from the empirical formula. From these results it could be concluded with certainty that biotin possessed a carboxylic acid group.

2. The Presence of a Urea Ring in Biotin

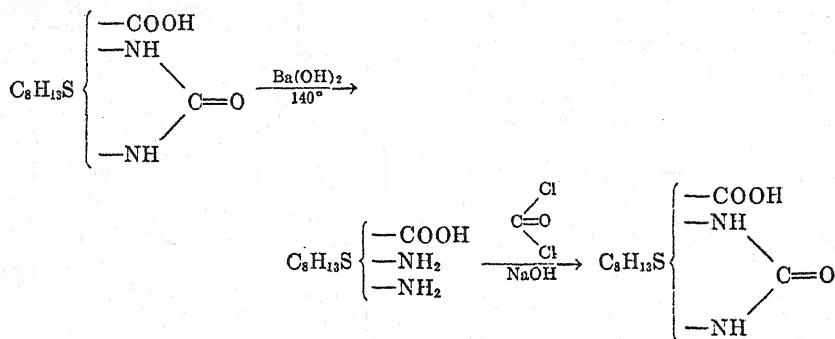
From the fact that biotin could be crystallized from strongly acid solutions, and from the shape of the titration curve for biotin, it appeared

evident that the basic group or groups present in the molecule were very weak. It was found that although biotin was inactivated rapidly by the Van Slyke amino-nitrogen procedure, no gas was formed, and no color was produced with ninhydrin (49). These results were in agreement with the non-inactivation of biotin with ninhydrin (6), and demonstrated that biotin did not possess an α -amino acid structure. A spectrographic study of biotin methyl ester in alcohol solution and of biotin in dilute alkali showed no specific absorption bands in the region examined, 2200 to 6000 Å (49). Kögl and Pons (59) likewise found no specific absorption in the ultraviolet for biotin methyl ester.

Many experiments were carried out on small amounts of pure biotin in an effort to determine the nature of the nitrogen atoms. Success was achieved finally by alkaline hydrolysis. Hofmann, Melville, and du Vigneaud (49) in 1941 described the formation of a sulfur-containing diaminocarboxylic acid by hydrolysis of biotin or its methyl ester with barium hydroxide at 140° for 20 hours. From 10 mg. of biotin there were obtained 10 mg. (85 per cent yield) of a compound which by analysis was shown to possess the empirical formula $C_9H_{20}O_6N_2S_2$. The pure compound, m.p. 245–255°, was optically active, $[\alpha]_D^{22} = -15^\circ$ for a 1 per cent solution in water. This compound was the sulfate of the base $C_9H_{15}O_2N_2S$, m.p. 186–190°, which was obtained from the sulfate by treatment with the calculated amount of barium hydroxide, and was purified by sublimation *in vacuo*. A micro Van Slyke amino-nitrogen determination on this compound indicated the presence of two primary amino groups. Treatment of the hydrolysis product with benzoyl chloride and alkali by the Schotten-Baumann method yielded a crystalline dibenzoyl derivative, $C_{23}H_{26}O_4N_2S$ m.p. 182–183°. The action of diazomethane on this derivative produced the methyl ester, $C_{24}H_{28}O_4N_2S$. The formation of the base $C_9H_{15}O_2N_2S$ from biotin ($C_{10}H_{16}O_3N_2S$) by alkaline hydrolysis had involved the loss of one carbon and one oxygen atom and a gain of two hydrogen atoms. Furthermore the weakly-basic biotin molecule had been converted to a relatively strong diacidic base which still possessed a carboxyl group. The most straight-forward interpretation of this reaction was the hydrolysis of a cyclic urea derivative to a diaminocarboxylic acid.

Proof that the above interpretation was correct was demonstrated by Melville, Hofmann, and du Vigneaud (71) by the resynthesis of biotin from the hydrolysis product. A solution of 10 mg. of the hydrolysis product in 1 cc. of 2 *N* NaOH was treated with gaseous phosgene in excess. Pure biotin crystallized from the cooled acid solution in almost quantitative yield. The biotin so obtained possessed the same melting point as that isolated from natural sources, and a mixture of the two showed no depression of the melting point. The specific rotation of the resynthesized compound was the same as that of natural biotin, which showed that no

racemization had occurred during the alkaline hydrolysis. Treatment of the reaction product with diazomethane yielded a methyl ester which was identical with the methyl ester of natural biotin. The yeast-growth activity of the resynthesized product was identical with that of natural biotin. This reaction therefore afforded additional and conclusive proof of the cyclic urea structure of biotin. The formulas could be written as follows:



In a paper which became available in this country shortly after the above work had been submitted for publication K \ddot{o} gl and Pons (59) described experiments in which they independently arrived at the same hydrolysis product of biotin, but by treatment of biotin with concentrated HCl at 200° for 1 hour. By comparison of the stability toward acid and alkali of ethylene urea and trimethylene urea derivatives with the stability of the urea group of biotin these authors were led to believe that biotin was probably a trimethylene urea derivative.

3. The Nature of the Sulfur of Biotin

The sulfur of biotin was found to be stable to vigorous treatment with alkali. No H₂S was liberated when biotin was treated with zinc dust and HCl, and after treatment of biotin with bromine water no inorganic sulfate could be detected. The nitroprusside test for sulfhydryl groups was negative. The absence of a low-molecular-weight alkyl sulfide group was indicated by the failure to obtain volatile alkyl iodide after vigorous treatment of biotin with HI. Positive evidence for the type of sulfur linkage present was afforded by the preparation of the sulfone of biotin (49), which was first suggested by the observed inactivation of biotin methyl ester by peroxide-containing ether. Accordingly, pure biotin was dissolved in glacial acetic acid, excess hydrogen peroxide was added, and the mixture was allowed to stand at room temperature for 18 hours.

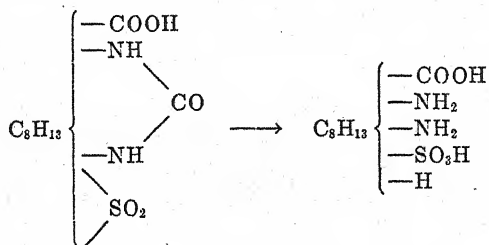
From the reaction mixture there was isolated in good yield a compound which by analysis was found to possess an empirical formula of $C_{10}H_{16}O_5N_2S$. This formula corresponds to biotin with two extra oxygen atoms. That the compound was the expected sulfone of biotin was indicated by color tests with tetranitromethane. This reagent produces a yellow color with unsaturated compounds, including thio ethers, the sulfur atom of which can be regarded as possessing a type of unsaturation. In sulfones, on the other hand, the sulfur atom is saturated, and no color is produced. It was found that while biotin produced a yellow color with tetranitromethane, the oxidation product yielded no color. The color produced by biotin must have been due to the sulfur atom since no ethylenic linkages appeared to be present in biotin, as judged by the recovery of unchanged biotin from catalytic and chemical reduction experiments. It seemed very likely, therefore, that the sulfur of biotin was present in a thio ether linkage.

Kögl and de Man (57) by titration of biotin with permanganate solution found that only two atoms of oxygen were readily taken up. From this fact they concluded that the sulfur of biotin was present as a thio ether. They succeeded in isolating the oxidation product and described it as biotin sulfone. Although no analyses were carried out on the compound it appears to be identical with the sulfone obtained independently by Hofmann, Melville, and du Vigneaud (49).

Consideration of the empirical formula of biotin and the types of groups present—carboxyl, cyclic urea, and thio ether—leads either to a molecule containing one ring and an ethylenic linkage, or to a bicyclic saturated molecule. In view of the negative results of attempted reductions of biotin it seemed unlikely that biotin contained an ethylenic bond. It appeared most probable therefore that biotin contained a bicyclic ring system, and failure to obtain any volatile alkyl iodide by vigorous treatment of biotin with HI indicated the possibility that the sulfur atom might be a member of one of the rings. What appeared to be the first direct evidence of this nature was presented in the paper by Kögl and de Man, and this will now be considered.

Kögl and de Man (57) described the hydrolysis of biotin sulfone with concentrated HCl at 200° for $\frac{1}{2}$ hour. By concentration of the hydrolysis mixture they obtained a hygroscopic, crystalline residue which yielded crystalline derivatives with picrolonic acid and dilituric acid (5-nitro-barbituric acid). A nitrogen analysis on the picrolonate, and carbon-hydrogen, nitrogen, and sulfur analyses on the diliturate indicated a composition of $C_8H_{20}O_5N_2S$ for the hydrolysis product, and this corresponds to the compound which would be formed by hydrolysis of the urea linkage

of biotin sulfone to a diamine, accompanied by hydrolytic splitting of a carbon-sulfur bond of the sulfone group to yield a sulfonic acid:



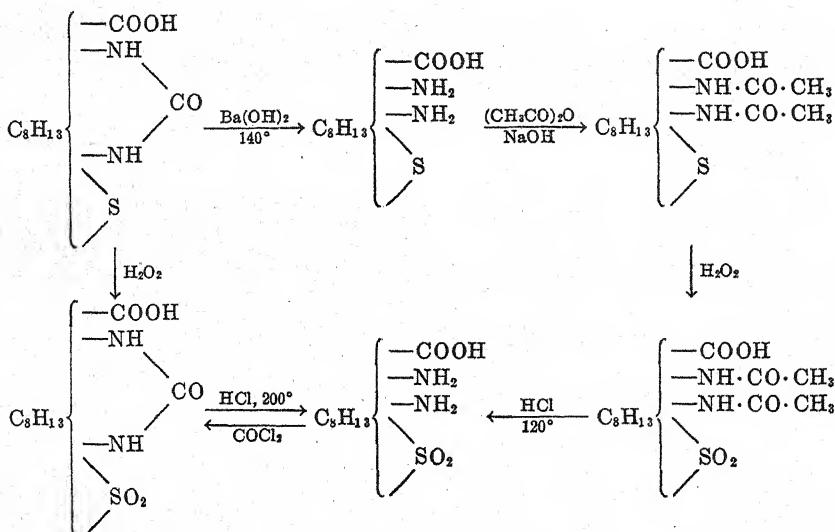
Substantiating evidence for the formation of a diaminocarboxysulfonic acid was offered by Kögl and de Man in a study of the electrometric titration curve of the hydrolysis product. Although the crystalline hydrolysis product, which they assumed to be the dihydrochloride, was not obtained in a pure state, Kögl and de Man found that almost exactly two equivalents of alkali were required to produce a steep rise in the titration curve of the compound, as compared with the requirement of only one equivalent of alkali to produce a similar rise when the dihydrochloride of the diaminocarboxylic acid from biotin was titrated. The extra equivalent of alkali apparently was required to titrate the sulfonic acid group in the hydrolysis product of the sulfone. If such a diaminocarboxysulfonic acid were indeed formed, without the loss of any of the carbon atoms of the diaminocarboxylic acid, it would be the first direct proof of the presence of the sulfur atom in a ring system. Other work, however, cast doubt on the correctness of the proposed structure of the hydrolysis product.

At Cornell Medical College attempts were being made to prepare the sulfone of the diaminocarboxylic acid derived from biotin. Treatment of biotin sulfone with $\text{Ba}(\text{OH})_2$, under conditions which had been used successfully in the preparation of the diaminocarboxylic acid from biotin itself, failed in this case. Since these results indicated the diaminocarboxylic acid sulfone was unstable toward alkali, direct oxidation of the sulfate of the diaminocarboxylic acid with H_2O_2 in glacial acetic acid was attempted. This reaction also failed to yield the desired compound; under the same conditions in which biotin is oxidized to the sulfone the diaminocarboxylic acid yielded a compound which analyzed correctly for the sulfoxide. Support for the sulfoxide structure was obtained by subjecting the compound to reduction with Zn and HCl, followed by treatment of the reaction mixture with alkali and phosgene. From the phosgene reaction mixture biotin was obtained in excellent yield.

More vigorous treatment of the diaminocarboxylic acid sulfate with H_2O_2 was not satisfactory for the preparation of the diaminocarboxylic acid sulfone. It was therefore decided to prepare the compound by oxidation of the diacetyldiaminocarboxylic acid, in which the amino groups are protected, followed by removal of the acetyl groups by acid hydrolysis. The diaminocarboxylic acid was acetylated in good yield with acetic anhydride and NaOH . Oxidation of the diacetyl derivative with H_2O_2 in glacial acetic acid gave the sulfone of the diacetyldiaminocarboxylic acid. The acetyl groups were removed, as shown by a micro Van Slyke amino-nitrogen determination, by hydrolysis with concentrated HCl at 120° for 1 hour. From the hydrolysis mixture there was obtained a crystalline, slightly hygroscopic hydrochloride, m.p. $142\text{--}152^\circ$, which could be best recrystallized from concentrated HCl .

Consideration of the work of Kögl and de Man made it seem possible that this hydrolysis product might be the same one obtained by those workers from biotin sulfone by hydrolysis with concentrated HCl at 200° for $\frac{1}{2}$ hour. Accordingly the hydrolysis of biotin sulfone was carried out under the conditions described by Kögl and de Man; by this treatment and by recrystallization from concentrated HCl a crystalline product was obtained in excellent yield which indeed appeared to be identical in all its properties with the hydrolysis product from the diacetyldiaminocarboxylic acid sulfone. Furthermore the dilituric acid derivatives prepared from both compounds appeared to be identical with one another and possessed the melting point ($235\text{--}240^\circ$) reported by Kögl and de Man for the diliturate of their hydrolysis product. It seemed probable that the hydrolysis product obtained at Cornell Medical College was the same compound obtained by Kögl and de Man.

The analytical values obtained on the compound, however, agreed more closely with those for the dihydrochloride of the diaminocarboxylic acid sulfone rather than for the dihydrochloride of a diaminocarboxysulfonic acid. In addition, the dihydrochloride, when titrated electrometrically, required exactly 3 equivalents of alkali to reach the inflection point on the steep part of the curve. Conclusive proof that the compound was a sulfone rather than a sulfonic acid derivative was given by Melville, Hofmann, and du Vigneaud (72) who showed that on treatment with alkali and phosgene it could be converted to biotin sulfone in 95 per cent yield. Because of the lability of the diaminocarboxylic acid sulfone to alkali best results were obtained by carrying out the reaction in carbonate solution at 0°C . The complete series of reactions can be formulated as follows:



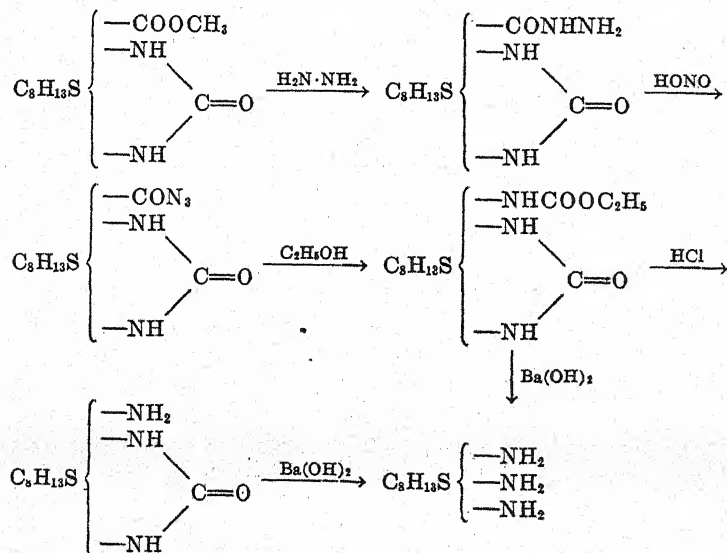
In view of the costliness of material no thorough attempt was made to explain the apparent divergence in results between the two laboratories. However, it was found that the dihydrochloride readily lost HCl. Evaporation to dryness over KOH of a water solution of the analytically pure compound yielded a higher-melting crystalline material which required only $2\frac{1}{2}$ equivalents of alkali for titration. The basicity of the amino groups apparently is greatly decreased when the thio ether atom is oxidized to a sulfone. It is possible that Kögl and de Man had prepared a monohydrochloride, since no analyses were performed, and titration of this compound would require 2 equivalents of alkali; one for the carboxylic acid group and one for the loosely-bound HCl. On the other hand, the dihydrochloride of the diaminocarboxylic acid requires only 1 equivalent of alkali for titration (for the carboxylic acid group) since the two HCl radicals are in salt formation with strongly basic amino groups.

4. The Presence of a Valeric Acid Side Chain in Biotin

While all the foregoing work had established with certainty the nature of the functional groups and hetero atoms of biotin, no positive evidence for the type of carbon skeleton present in the molecule had been obtained, aside from the knowledge that biotin contained one ring system and the probability that it contained two. The first evidence in this direction was described by Hofmann, Melville, and du Vigneaud (48, 50). It was found that oxidation of the diaminocarboxylic acid from biotin with either alkaline permanganate or with concentrated nitric acid yielded an ether-soluble acidic oxidation product which after crystallization from ether melted at $152\text{--}153^\circ$. From the oxidation of 10 mg. of the diamino-

carboxylic acid sulfate with nitric acid, followed by continuous ether extraction of the oxidation mixture, sublimation of the ether soluble material, and crystallization of the sublimate from ether, there was obtained 1 mg. of the compound. A neutral equivalent of 73 was obtained; both this and the melting point indicated that the compound was adipic acid. A mixture of the isolated compound with an authentic sample of adipic acid showed no depression of the melting point. Furthermore, the diamide, m.p. 224–226°, and the di- β -naphthylamide, m.p. 267–268°, prepared from the oxidation product agreed in properties with the diamide and di- β -naphthylamide of adipic acid. Analytical values obtained on the di- β -naphthylamide of the oxidation product were in agreement with the calculated values. It appeared certain from these data that the oxidation product was adipic acid.

The adipic acid formed in these oxidations could have arisen either from an aliphatic acid side chain in biotin, or from a cyclic 6-carbon structure cleaved by the oxidation. In the former case the original carboxyl group of biotin would appear as one of the carboxyl groups of the adipic acid; in the latter case neither of the carboxyl groups of the adipic acid would be the original carboxyl group in biotin. Obviously, if the carboxyl group of biotin could be labelled or changed in some manner before the oxidation, it should be possible to differentiate between these two possibilities. After several attempts by other methods the objective was achieved by a Curtius degradation of biotin methyl ester (50). In this way the carboxyl group of biotin was replaced by an amino group, as indicated by the following formulas:

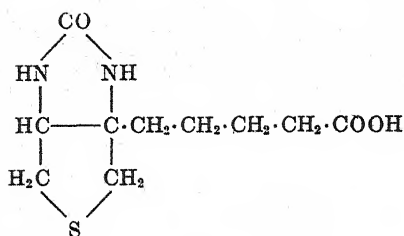


Biotin methyl ester was converted to the acid hydrazide, $C_{10}H_{18}O_2N_4S$, by heating with hydrazine hydrate at 130° for 3 hours. Treatment of the hydrazide with nitrous acid yielded the azide, which was converted to the urethane, $C_{12}H_{21}O_3N_3S$, by refluxing with ethanol. The urethane, in turn, was converted to the amine hydrochloride, $C_8H_{17}ON_3S \cdot HCl$, by heating with concentrated HCl for 2 hours on the steam bath. The preparation of the triamine, $C_8H_{19}N_3S$, was accomplished by hydrolyzing the urea group of the amine with $Ba(OH)_2$ at 140° . The compound was obtained as the sulfate-hydrate, $C_8H_{19}N_3S \cdot 1\frac{1}{2}H_2SO_4 \cdot H_2O$. The same compound was obtained directly from the urethane by hydrolysis with $Ba(OH)_2$ at 140° . Analyses of the triamine sulfate and of the tripicrolonate and tribenzoyl derivatives, the latter obtained by treatment with benzoyl chloride and KOH , all indicated the formula $C_8H_{19}N_3S$ for the free base.

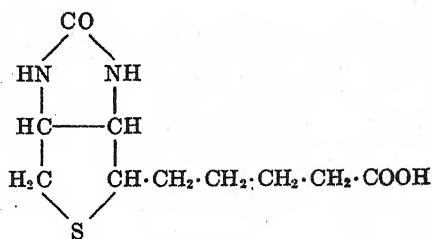
The triamine was subjected to the same oxidation procedures which had been employed for the oxidation of the diaminocarboxylic acid. In experiments in which 10 mg. of the triamine were oxidized with nitric acid no indication of the presence of adipic acid among the ether-soluble oxidation products was observed. Under the same conditions adipic acid had been readily isolated after the oxidation of similar amounts of the diaminocarboxylic acid. Finally, when sufficient material had become available, 50 mg. of the triamine sulfate were oxidized with alkaline permanganate; here again no trace of adipic acid could be detected, although the amount of adipic acid which might have been formed would have permitted of comparatively easy isolation and identification.

The non-formation of adipic acid by oxidation of the triamine is strong evidence—albeit negative evidence—that the adipic acid formed by oxidation of the diaminocarboxylic acid arises, not from a cyclic structure, but from an aliphatic acid side chain attached to a ring-carbon in the biotin molecule. On the strength of this evidence it was felt that the number of possible structures that could be ascribed to biotin was reduced sufficiently to warrant individual consideration of the possibilities.

Early in 1942, du Vigneaud, Hofmann, and Melville (16) published a note outlining the few structures which were in conformity with the experimental results so far obtained. It was felt that the marked stability of the diaminocarboxylic acid toward hydrolytic agents rendered unlikely structures in which the sulfur atom and an amino group were attached to the same carbon atom. Such compounds, where described in the literature, appear to be unstable toward acid and alkali. With this limitation set, the most logical interpretation of the data so far accumulated led to two structures containing a tetrahydrothiophene nucleus with a *n*-valeric acid side chain attached in the β or α position, as depicted in formulas I and II:

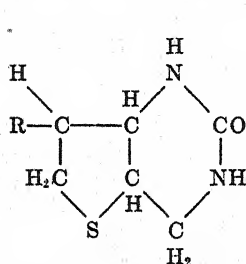


(I)

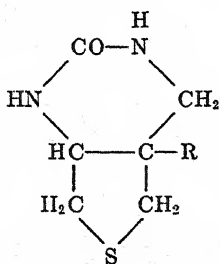


(II)

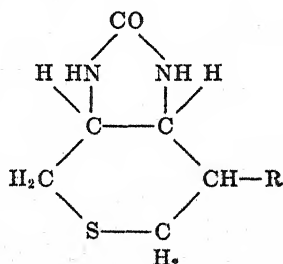
While either of these formulas would explain the formation of adipic acid by oxidation, it was possible that the adipic acid had arisen from the decarboxylation of a malonic or α -substituted β -keto acid arising during the oxidation. If such were the case, then three additional structures became possibilities, as shown in formulas III, IV, and V:



(III)



(IV)



(V)



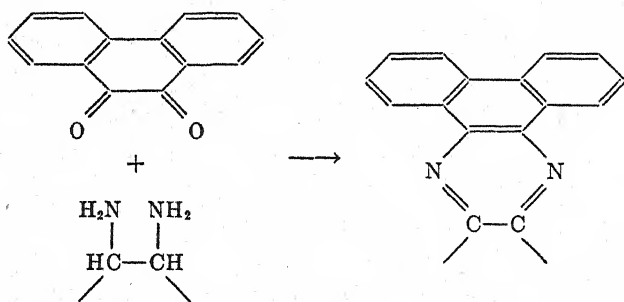
The possibility that an X-ray diffraction study might aid in eliminating one or more of the five structural possibilities was investigated. From this work, which was carried out by Fankuchen (27), it was shown that the biotin molecule possessed dimensions of approximately $5 \times 5.5 \times 10.5$ Å. However, none of the above five structures could be conclusively ruled out by the X-ray results.

5. The Size of the Urea Ring in Biotin

Of the five structures which agreed with the chemical data, three possessed 5-membered urea rings, while two were trimethylene urea derivatives. Obviously a reaction which would differentiate between an ethylene urea ring and a trimethylene urea ring; *i.e.*, between a 1,2-diamine and a 1,3-diamine in the diaminocarboxylic acid, would be of great value in further limiting the possible structures for biotin. It was found by Hofmann, Kilmer, Melville, du Vigneaud, and Darby (47) that the reaction of the diaminocarboxylic acid with phenanthrenequinone afforded such a means

of differentiation. Treatment of 14 mg. of the sulfate of the diaminocarboxylic acid with the calculated amount of $\text{Ba}(\text{OH})_2$ yielded the free base; this was heated under reflux in alcohol solution with 10 mg. of phenanthrenequinone for 14 hours. Crystals separated from the cooled solution. Two recrystallizations from ethanol-water yielded 8 mg. of pale yellow needles, m.p. $202\text{--}204^\circ$. Analyses of this compound suggested the empirical formula $\text{C}_{23}\text{H}_{20}\text{O}_2\text{N}_2\text{S}$. The compound gave a red color with concentrated sulfuric acid, and in benzene solution showed a strong blue fluorescence.

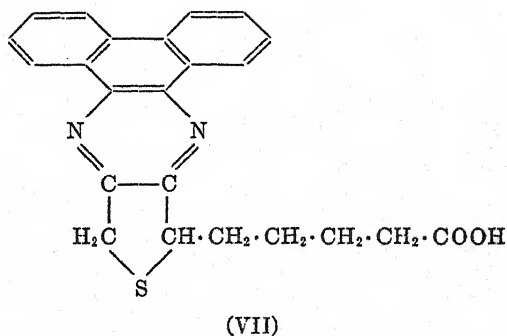
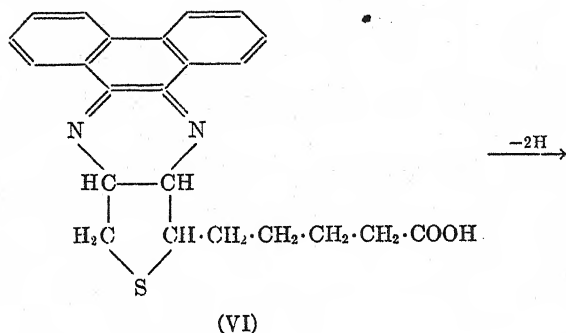
While the condensation of phenanthrenequinone with 1,2-diamines is a general method for forming dibenzoquinoxalines



particularly with aromatic 1,2-diamines, no examples of the condensation of 1,3-diamines with phenanthrenequinone could be found in the literature. The formation of the condensation product of the diaminocarboxylic acid with phenanthrenequinone therefore afforded strong evidence that the diaminocarboxylic acid was a 1,2-diamine, and thus formulas III and IV were eliminated as possible structures for biotin, since biotin must contain a 5-membered urea ring.

The initial product expected from the condensation of phenanthrenequinone with an aliphatic 1,2-diamine would be a dihydroquinoxaline. However, the analyses of the condensation product obtained from the diaminocarboxylic acid indicated that the compound was a quinoxaline rather than a dihydroquinoxaline. The red color obtained by treatment of the compound with sulfuric acid also indicated a quinoxaline structure. Whether a dihydroquinoxaline or quinoxaline compound had been formed became of importance to the structure of biotin by consideration of the expected reactions of the diaminocarboxylic acids derived from the three remaining possible biotin structures (formulas I, II, and V). The diaminocarboxylic acid derived from the compound corresponding to either formula II or V could yield a dihydroquinoxaline which would be expected to be dehydrogenated rather readily to a quinoxaline. For example, the compound from formula II should be transformed from the dihydroquinoxaline (VI) to the quinoxaline (VII) as shown in the accompanying

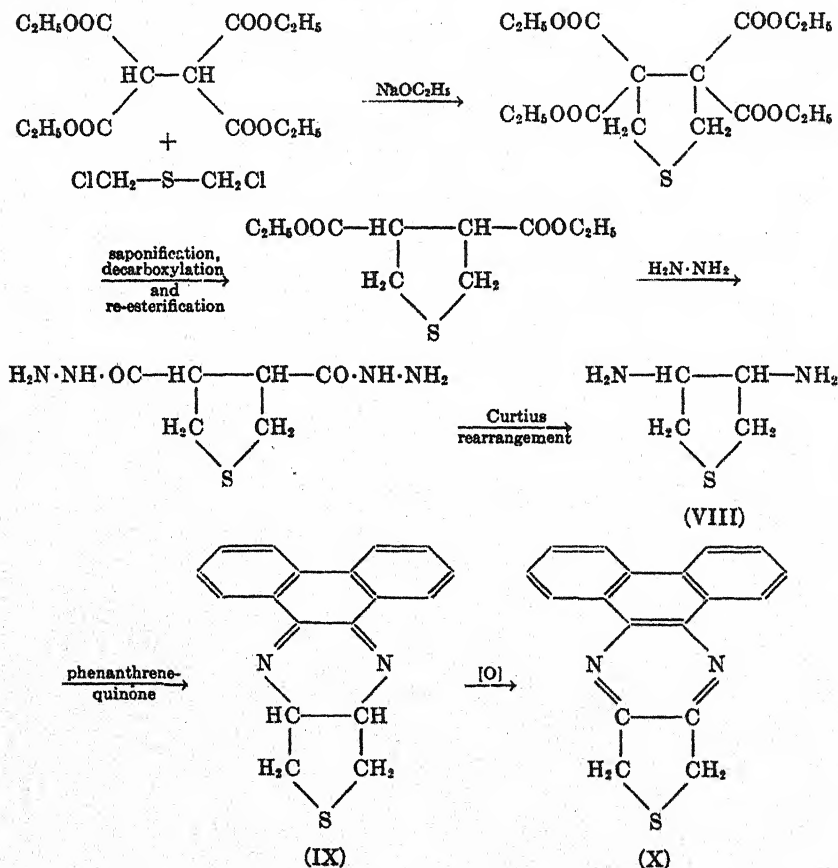
formulas. On the other hand, the dihydroquinoxaline corresponding to formula I would not be expected to undergo dehydrogenation under ordinary conditions because of the absence of a necessary hydrogen atom on the ring-carbon atom bearing both the side chain and one of the amino groups.



It was considered 'probable' that a study of the ultraviolet absorption spectrum of the condensation product would provide a means of distinguishing between the two forms, since the absorption of the dihydroquinoxaline should be different from that of the more fully aromatic quinoxaline. Consequently the absorption spectrum of the condensation product was compared with the absorption spectra of the quinoxaline and dihydroquinoxaline derivatives obtained by condensing phenanthrenequinone with 3,4-diaminotetrahydrothiophene. The synthesis of the 3,4-diaminotetrahydrothiophene has been described by Kilmer, Armstrong, Brown, and du Vigneaud (53). The method used is indicated by the formulas on page 54.

When the 3,4-diaminotetrahydrothiophene (VIII) was treated with phenanthrenequinone under conditions similar to those used with the diaminocarboxylic acid from biotin there was obtained a reddish-orange compound, m.p. 183-185°, which by analysis was shown to possess the empirical

formula $C_{18}H_{14}N_2S$. The substance was found to be the dihydroquinoxaline derivative (IX) as shown by analysis, by the formation of a green color with sulfuric acid, and by conversion to the quinoxaline derivative, $C_{18}H_{12}N_2S$. This compound (X) was obtained by heat-treatment of the dihydroquinoxaline in air at 200° and then sublimation. Crystallization of the sublimate from chloroform yielded pale yellow prisms of the pure compound, m.p. $228-233^\circ$, which gave a red color with sulfuric acid.



As had been anticipated, the quinoxaline and dihydroquinoxaline derivatives of the 3,4-diaminotetrahydrothiophene possessed distinctly different absorption spectra. In addition (Fig. 2) it was found that the absorption spectrum of the condensation product of phenanthrenequinone with the diaminocarboxylic acid from biotin was almost identical in shape with that of the quinoxaline derivative of the 3,4-diaminotetrahydrothio-

phene, and showed little resemblance to the spectrum of the dihydro form of the same compound. It was evident from these data that the condensation product from biotin contained the completely aromatic quinoxaline ring system, and therefore the diaminocarboxylic acid must possess a hydrogen atom attached to each of the carbon atoms holding the amino groups. The structure illustrated by formula I did not comply with this limitation and so was eliminated from consideration. Each of the two remaining possibilities (Formulas II and V) carries the requisite hydrogen

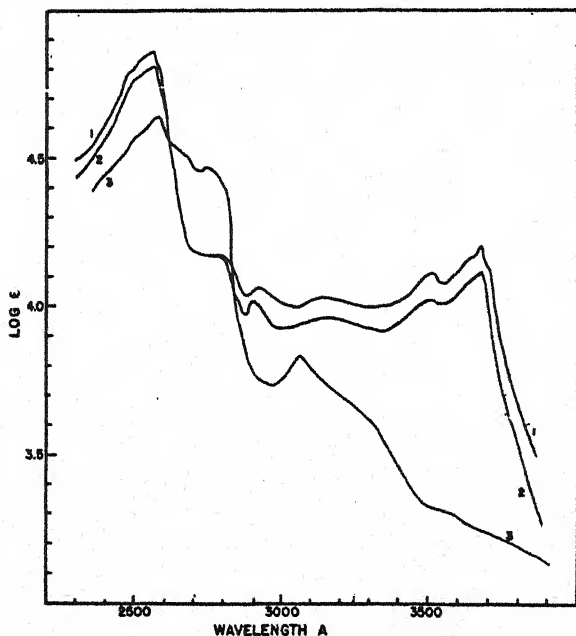


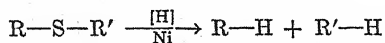
Fig. 2. Ultraviolet Absorption Spectra of the Condensation Product of Phenanthrenequinone with (1) the Diaminocarboxylic Acid from Biotin; (2) 3,4-Diaminotetrahydrothiophene, Oxidized Form; (3) 3,4-Diaminotetrahydrothiophene, Reduced Form

atoms adjacent to the amino groups. The final choice between these two alternatives rested on two independent methods of proof, each pointing unequivocally to one and only one of these structures as the correct structure for biotin

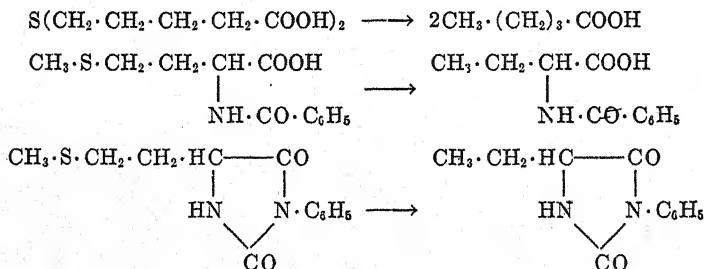
6. Desthiobiotin

The first method of final proof of structure which will be considered was based on a study of "desthiobiotin," the compound corresponding to biotin with the sulfur atom removed and replaced by two hydrogen atoms.

It was found that this compound could be prepared from biotin by the application of a desulfurization reaction for thio ethers devised by Mozingo, Wolf, Harris, and Folkers, which consisted of the treatment of organic sulfides in alcohol solution with large amounts of Raney nickel catalyst in the absence of a hydrogen atmosphere:



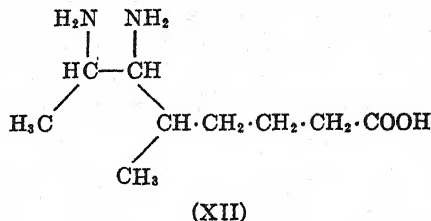
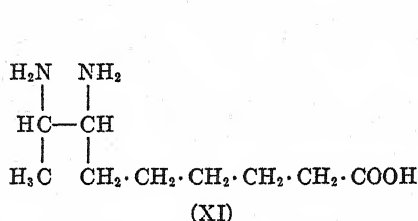
Although Bougault, Cattelian, and Chabrier (5) had previously shown that the sulfur could be removed from sulfhydryl compounds by treatment with Raney nickel, no examples of the reduction of organic sulfides in this manner had been described. By this method the sulfides shown in the accompanying formulas were cleaved to their corresponding sulfur-free products in yields of 65 to 90 per cent on both a macro and semi-micro scale.



As described by du Vigneaud, Melville, Folkers, Wolf, Mozingo, Keresztesy, and Harris (19), biotin methyl ester when treated under these conditions yielded desthiobiotin methyl ester, $\text{C}_{11}\text{H}_{20}\text{O}_3\text{N}_2$, which by hydrolysis with either $\text{Ba}(\text{OH})_2$ at 140° or concentrated HCl at 200° gave the desthiodiaminocarboxylic acid, $\text{C}_9\text{H}_{20}\text{O}_2\text{N}_2$. From 100 mg. of biotin methyl ester, after treatment under reflux in 90 per cent ethanol with 5 grams of Raney nickel for 5 hours, there were obtained 85 mg. of desthiobiotin methyl ester, m.p. $69\text{--}70^\circ$. By treatment of 100 mg. of this compound with $\text{Ba}(\text{OH})_2$ under the same conditions used for the hydrolysis of the urea ring of biotin, 151 mg. of desthiodiaminocarboxylic acid sulfate, m.p. $242\text{--}243^\circ$, were obtained.

By consideration of the two remaining possible biotin structures (formulas II and V) it is evident that the structure of the desthiodiaminocarboxylic acid obtained by desulfurization and then hydrolysis of the urea ring would be either ζ, η -diaminopelargonic acid (XI), if formula II represented the correct structure of biotin; or δ -methyl- ϵ, ζ -diaminocaprylic acid (XII), if biotin possessed the structure shown by formula V.

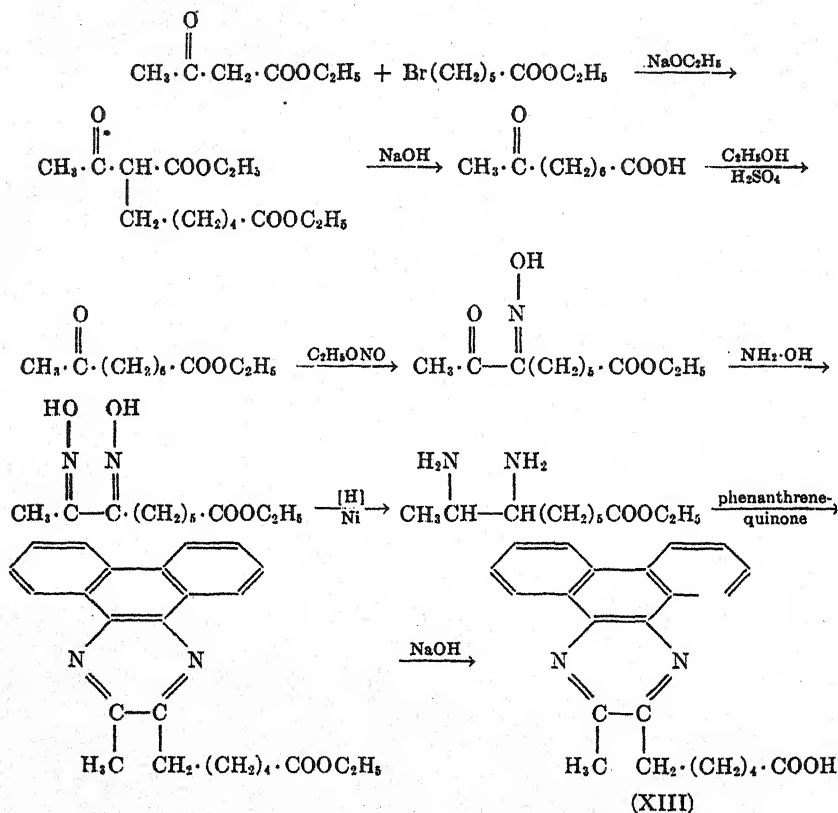
A Kuhn-Roth carbon-methyl determination should distinguish between these two possibilities, since the diamino acids XI and XII contain one and two carbon-methyl groups respectively. More positive characterization of the desthiodiaminocarboxylic acid would be afforded by an oxidative cleavage between the two carbon atoms bearing the amino groups. In the case of a structure corresponding to XI, pimelic acid should be formed, while from structure XII α -methyl adipic acid should result.



A carbon-methyl determination on the desthiodiaminocarboxylic acid sulfate gave a value of 1.88 per cent as compared with the theoretical value of 5.25 per cent for one carbon-methyl group. Oxidation of the desthiodiaminocarboxylic acid with alkaline permanganate and with nitric acid in each case gave low yields of mixtures of adipic and pimelic acids. Unsatisfactory results were also obtained when lead tetraacetate or alkaline hypochlorite were used. However, oxidation with alkaline periodate gave a good yield of crude acid from which pure pimelic acid was obtained. From 50 mg. of the desthiodiaminocarboxylic acid sulfate an ether-soluble fraction weighing 23 mg. was obtained. Three such fractions, combined and purified by sublimation *in vacuo* and crystallization from ether + petroleum ether, yielded approximately 17 mg. of pure pimelic acid, $\text{C}_7\text{H}_{12}\text{O}_4$, m.p. 103–104°. The pimelic acid was identified by analysis, a mixed melting point, and by the preparation of the di-*p*-bromophenacyl ester, which agreed in all its properties with an authentic sample of di-*p*-bromophenacyl pimelate.

Further characterization of the desthiodiaminocarboxylic acid was accomplished by complete synthesis of the dibenzoquinoxaline derivative of the compound, by the method outlined in the formulas on the following page (19).

The synthetic quinoxaline derivative (XIII) was a pale yellow solid, m.p. 186–187°, which by analysis was found to possess the empirical formula $\text{C}_{22}\text{H}_{22}\text{O}_2\text{N}_2$. That the compound was indeed the quinoxaline and not the dihydroquinoxaline was shown by the formation of a red color with sulfuric acid and by the ultraviolet absorption spectrum of the compound, which was similar to that of the quinoxaline derivative of 3,4-diaminotetrahydrothiophene (X).



Consideration of formula XIII shows that in the formation of this compound from ζ, η -diaminopelargonic acid the asymmetry of the two carbon atoms bearing the amino groups is destroyed; the resulting compound (XIII) possesses no centers of asymmetry. It was for this reason that the quinoxaline derivative of the synthetic diaminopelargonic acid was prepared to compare with the corresponding derivative of the desthiodiaminocarboxylic acid from biotin. In this way resolution of the synthetic diamino acid, which would have been necessary for direct comparison with the desthiodiaminocarboxylic acid, was obviated. In addition, if formula V were the correct structure for biotin, then the quinoxaline prepared from the corresponding desthiodiamino acid (XII) would still possess an asymmetric carbon atom, and therefore the product would be expected to show optical activity. Thus an additional and convenient method of differentiation between the two possibilities was available.

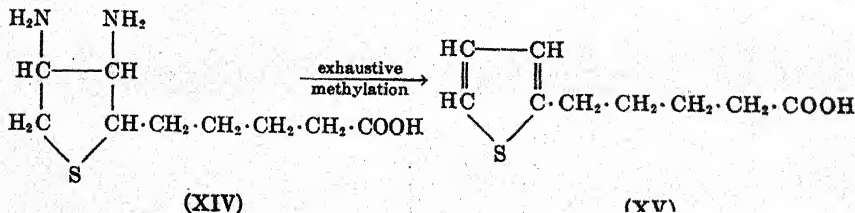
The desthiodiaminocarboxylic acid from biotin was converted to the quinoxaline derivative by condensation with phenanthrenequinone under the same conditions that had been used for the condensation of the diamino-

carboxylic acid with this reagent. A pale yellow condensation product, $C_{23}H_{22}O_2N_2$, m.p. $186-187^\circ$, was isolated in good yield from the reaction mixture. This compound appeared to be identical in all its properties with the quinoxaline derivative of the synthetic diamino acid (XIII). A mixture of the two compounds showed no depression of the melting point. Furthermore, the quinoxaline derivative of the desthiodiaminocarboxylic acid from biotin showed no optical activity. It is obvious from these data that of the two possible structures (II and V) the only structure of biotin which agreed with the facts observed in the investigation of desthiobiotin was that corresponding to formula II. Another type of evidence which likewise provided proof of the correctness of formula II will now be considered.

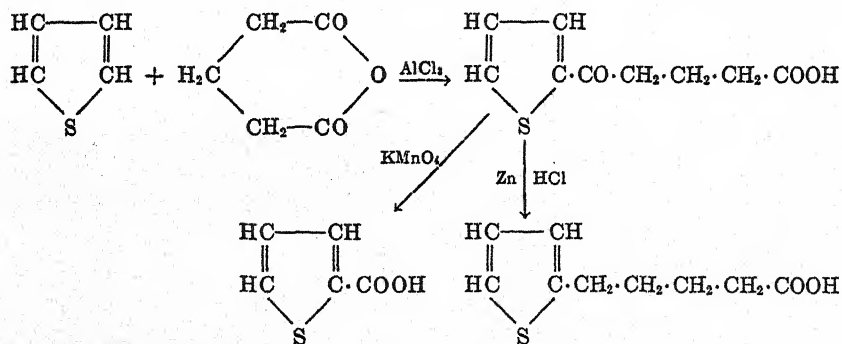
7. The Formation of Thiophenevaleric Acid from Biotin

Direct proof of the presence of a five-membered sulfur ring in biotin, with a *n*-valeric acid side chain attached in the α -position to the sulfur, was obtained by Melville, Moyer, Hoffmann, and du Vigneaud (74) by exhaustive methylation studies on the diaminocarboxylic acid from biotin. Exhaustive methylation as a method of degradation for the diaminocarboxylic acid was investigated soon after the compound was first prepared, but preliminary experiments with methyl iodide and with dimethyl sulfate yielded no isolable products. Kögl and de Man (57) likewise were unable to obtain satisfactory results by exhaustive methylation of the diaminocarboxylic acid with methyl iodide and silver oxide.

After the demonstration that biotin contained a 4- or 5-carbon aliphatic side chain (16), and that the amino groups were attached to adjacent carbon atoms, each of which bore a hydrogen atom (47), the possible structures had been limited to formulas II and V. It was felt that the mechanism of formation of adipic acid from biotin by oxidation could be explained more reasonably by the oxidation of a *n*-valeric acid side chain attached to a ring-carbon atom, rather than by the intermediary formation and decarboxylation of an α -substituted β -keto acid or a malonic acid. Thus the favored structure was that expressed by formula II, and it was felt that, by exhaustive methylation of the diaminocarboxylic acid corresponding to this structure (XIV) it should be possible to obtain a thiophene derivative, as indicated in the accompanying formulas. In the case of a structure such as V no thiophene derivative would be anticipated.



The expected product, δ -(α -thienyl)-valeric acid (XV), could be easily synthesized in the laboratory for comparative purposes. On the strength of the belief that formula II did represent the true structure of biotin, and that it should be possible to obtain XV by exhaustive methylation of the diaminocarboxylic acid, this thiophene derivative was synthesized for future comparative purposes, by the method used by Fieser and Kennelly (28) for the synthesis of the lower homolog, γ -(α -thienyl)-butyric acid. The procedure used is shown in the accompanying formulas; the position of the side chain in the synthetic product was determined as indicated in the formulas by oxidation of the intermediary γ -(α -thienyl)-butyric acid with alkaline permanganate to α -thiophenic acid, m.p. 125–126°, a known compound. The γ -(α -thienyl)-butyric acid, m.p. 92–94°, from the Friedel-Crafts reaction between thiophene and glutaric anhydride was reduced by the Clemmensen method. Distillation of the reduction product gave pure δ -(α -thienyl)-valeric acid, m.p. 40–41°.



When sufficient biotin became available, methylation of the diaminocarboxylic acid from biotin was again attempted, but on a larger scale than the previous experiments. Preliminary experiments in which small amounts of 3,4-diaminotetrahydrothiophene (VIII) were methylated with methyl iodide and with dimethyl sulfate indicated that dimethyl sulfate was the more satisfactory reagent for the formation of thiophene from this compound (as judged by the amount of color produced when the distillate obtained by heating the alkaline methylation mixture was treated with isatin-sulfuric acid reagent). Consequently a solution of 50 mg. of the diaminocarboxylic acid sulfate from biotin was treated with a slight excess of dimethyl sulfate and KOH in successive small amounts. Attempts to isolate a crystalline methylated compound from the reaction mixture failed. Furthermore, decomposition of the methylation mixture at various temperatures and under varying degrees of alkalinity yielded much trimethylamine but produced no isolable amounts of material of the desired chemical and physical characteristics. However, it was found that a methylation

mixture which had been refluxed with HCl (to decompose any metho-sulfate salt) contained 2 mg. of an ether-soluble oil which on fractional distillation *in vacuo* yielded 1 mg. of an acidic, sulfur-containing crystalline compound, m.p. 40–41°. From several experiments a total of 5 mg. of the pure compound was obtained.

Variations in the conditions of methylation did not increase the yield of the crystalline compound. Attempts to prepare the compound in better yield by other methods of degradation were likewise unsuccessful. These procedures included distillation of the phosphate salt of the diaminocarboxylic acid by the method described by Harries (42); methylation with formaldehyde + formic acid (11) followed by pyrolysis of the methylated product, in view of the ease of formation of divinyl sulfide from bis-(β -dimethylaminoethyl)-sulfide (65); and methylation of the sulfoxide of the diaminocarboxylic acid, followed by reduction and pyrolysis. The failure of the decomposition of a methylated diaminocarboxylic acid of a structure corresponding to XIV to produce large amounts of the expected product (XV) is possibly not surprising in the light of the results obtained by Buchman, Schlatter, and Reims (7) who found that the thermal decomposition of *trans*-1,2-cyclobutane-bis-(trimethylammonium) hydroxide yielded none of the expected cyclobutadiene, and by Hurd and Drake (51) who showed that conjugated unsaturation does not result exclusively during the pyrolysis of 1,2-bis-(trimethylammonium) hydroxides. In addition, it is probable that methylation of the thio ether by dimethyl sulfate also occurs, and the resulting sulfonium compound could decompose in more than one way. Although Kögl and de Man (57) found that biotin methyl ester on treatment with methyl iodide readily yielded a methyl sulfonium compound which decomposed smoothly to the original sulfide (biotin methyl ester), it is quite possible that the sulfonium derivative of the diaminocarboxylic acid or of the *N*-methylated diaminocarboxylic acid would decompose in a different manner. In this respect it should be noted that the methyl sulfonium base of α -methyltetrahydrothiophene readily undergoes ring fission (41), and the corresponding sulfonium derivative of tetrahydrothiophene appears to follow the same path of decomposition (105).

The small amount of crystalline compound isolated from the methylation experiments possessed the same crystalline form and melting point (40–41°) as the synthetic δ -(α -thienyl)-valeric acid. A mixture of the two compounds showed no depression of the melting point. Both compounds gave a slowly-forming purple color with isatin-sulfuric acid reagent, and a deep blue-green color with Liebermann's nitrite-sulfuric acid reagent. Analyses of the isolated compound were in agreement with the calculated values for thiophenevaleric acid. The concluding proof of the structure of the isolated compound was obtained by a comparison of the ultraviolet absorption spectra of the isolated compound and the synthetic δ -(α -thienyl)-valeric

acid. As shown in Fig. 3, in which the wave length is plotted against the specific extinction coefficient, both compounds showed identical absorption curves, with a maximum absorption at 234 m μ . The difference in heights of the absorption peaks may be due in large part to the experimental error in weighing the small sample of isolated compound (0.173 mg.).

This demonstration of the formation of δ -(α -thienyl)-valeric acid from the diaminocarboxylic acid from biotin, under conditions which would not be expected to produce any deep-seated rearrangement, presented, in conjunction with the results obtained by the study of desthiobiotin, very strong

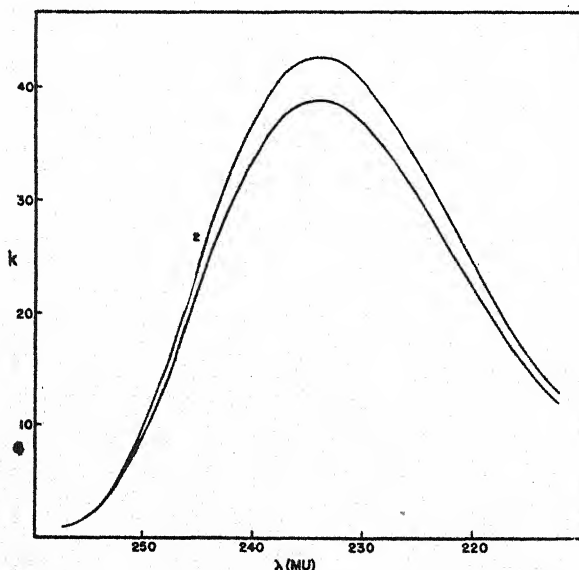
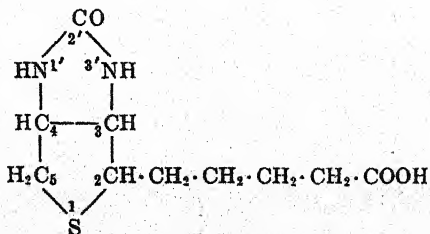


Fig. 3. Ultraviolet Absorption Spectra of (1) the Compound Isolated from Biotin; (2) Synthetic δ -(α -Thienyl)-valeric acid, in 95 per cent Alcohol. The wave length is plotted against the specific extinction.

evidence that the correct structure of biotin is that represented by the following formula:



Biotin

(2'-Keto-3,4-imidazolido-2-tetrahydrothiophene-*n*-valeric acid)

Recently Harris, Wolf, Mazingo, and Folkers (43) have described the properties of biotin obtained by synthetic methods. The synthetic compound possessed the same crystalline form, solubilities, and melting point as the natural product. The specific rotation, $[\alpha]_D^{25} = +90.7^\circ$, was in good agreement with the value obtained with natural biotin. Analytical values agreed with the empirical formula $C_{10}H_{16}O_3N_2S$. The physiological effects of the synthetic compound were found to be identical with natural biotin for the growth of *Lactobacillus arabinosus* and the cure of the egg white injury syndrome in rats and chicks. Although the method of synthesis was not disclosed, the results may be taken as complete confirmation of the structure assigned to biotin by du Vigneaud and co-workers (14a).

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ADDENDUM

Very recently there has become available a series of papers published by Kögl and his coworkers concerning further degradative studies on biotin isolated from egg yolk, and a comparison of biotin from egg yolk and from liver. By a procedure similar to that employed by the American workers, Kögl and ten Ham (119, 120) succeeded in isolating from liver sufficient crystalline material for purposes of comparison. Based on melting points, mixed melting points, and specific rotations of biotin and biotin methyl ester from egg yolk and liver, it was concluded that the two compounds were not identical. The compounds from egg yolk and liver were designated " α -biotin" and " β -biotin," respectively. The data obtained by Kögl are summarized in Table I (p. 67).

For comparison, Table II lists the corresponding data obtained at Cornell Medical College on biotin from liver and milk; biotin obtained from two other sources was found to be identical with that from liver and milk. It should be noted that the specific rotations of biotin methyl ester in Table II and β -biotin methyl ester in Table I differ quite markedly.

Kögl stated that a mixture of the α - and β -esters showed a melting-point depression of 20–30°; a mixture of the free acids melted at 197–202°. X-ray analyses confirmed the non-identity of the two substances. The β -biotin possessed approximately twice the yeast-growth activity of α -biotin. β -Biotin was believed to be identical with the biotin studied by the American workers, in spite of differences in optical activity of the esters.

By subjecting the diaminocarboxylic acid derived from α -biotin isolated from egg yolk, to oxidation with lead tetraacetate, Kögl, Erxleben, and Verbeek (118) were able to isolate an aldehyde, $C_8H_{15}O_2NS$, as the dinitrophenylhydrazone derivative. Oxidation of the aldehyde with $KMnO_4$, and treatment of the oxidation mixture with methanol-HCl yielded an

unsaturated, nitrogen-free ester. By treatment of this ester with KMnO_4 , a sulfocaproic acid, $\text{C}_6\text{H}_{12}\text{O}_6\text{S}$, was obtained as the crystalline *m*-toluidine salt, $\text{C}_{13}\text{H}_{21}\text{O}_6\text{NS}$. By alkaline fusion of the sulfocaproic acid Kögl, Ver-

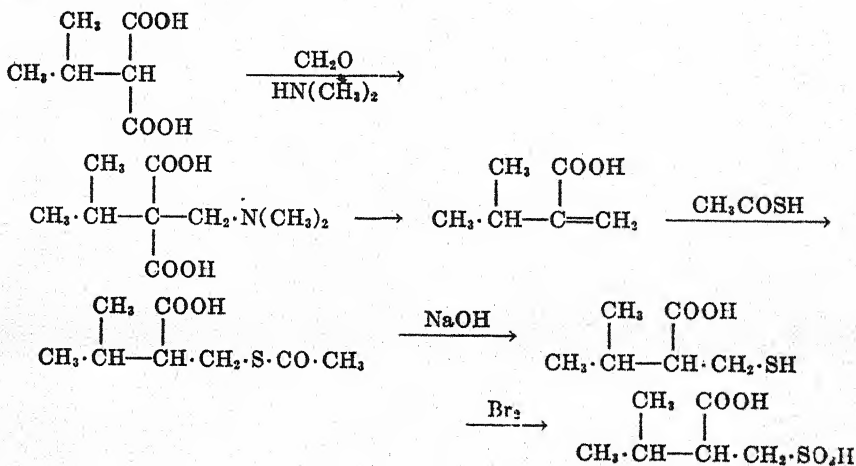
TABLE I

Substance	Micro-m.p. (corrected)	Optical Activity	
		$[\alpha]_D$	Solvent
α -Biotin methyl ester	161-162°	+47° +82° \pm 3°	Chloroform Methanol
β -Biotin methyl ester	163-164°	+39°	Chloroform
α -Biotin	220°	+51°	0.1 N NaOH
β -Biotin	232-233°	+91°	0.1 N NaOH

TABLE II

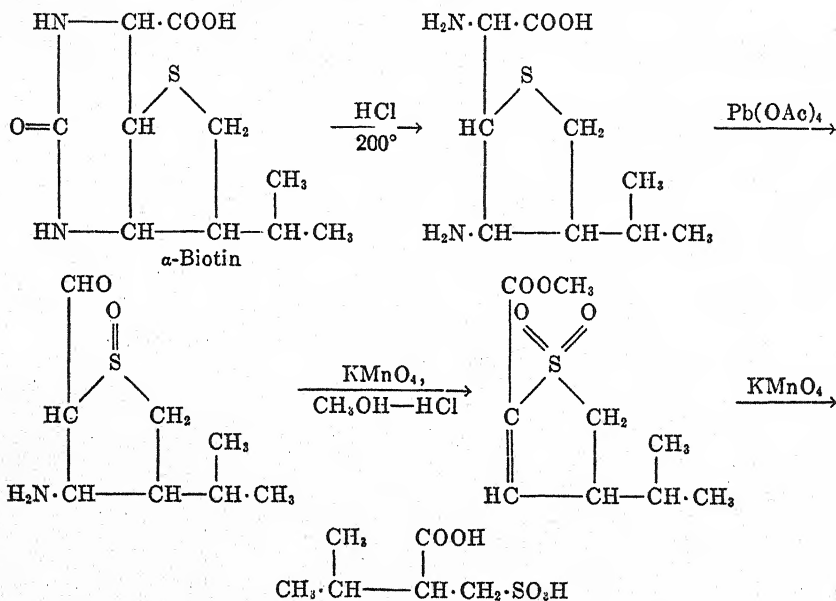
Substance	Micro-m.p. (corrected)	Optical Activity	
		$[\alpha]_D$	Solvent
Biotin methyl ester	162-163°	+57° +79°	Chloroform Methanol
Biotin	229-230°	+92°	0.1 N NaOH

beek, Erxleben, and Borg (121) obtained an unsaturated acid which on hydrogenation yielded α, β -dimethylbutyric acid, characterized as the *p*-phenylphenacyl ester. Synthetic α -isopropyl- β -sulfopropionic acid, prepared as indicated,



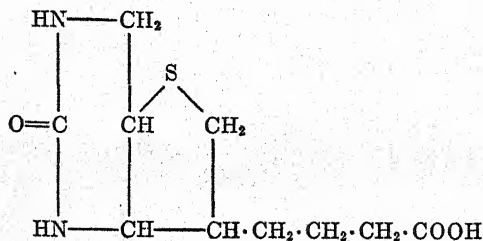
was resolved and the (–) form was found to give a *m*-toluidine salt and an anilide of the sulfomethyl ester which were identical, as determined by melting points and mixed melting points, with the corresponding derivatives obtained from the sulfocaproic acid from α -biotin.

On the basis of this work, the structure of α -biotin, and its degradation to α -isopropyl- β -sulfopropionic acid, were formulated as follows:



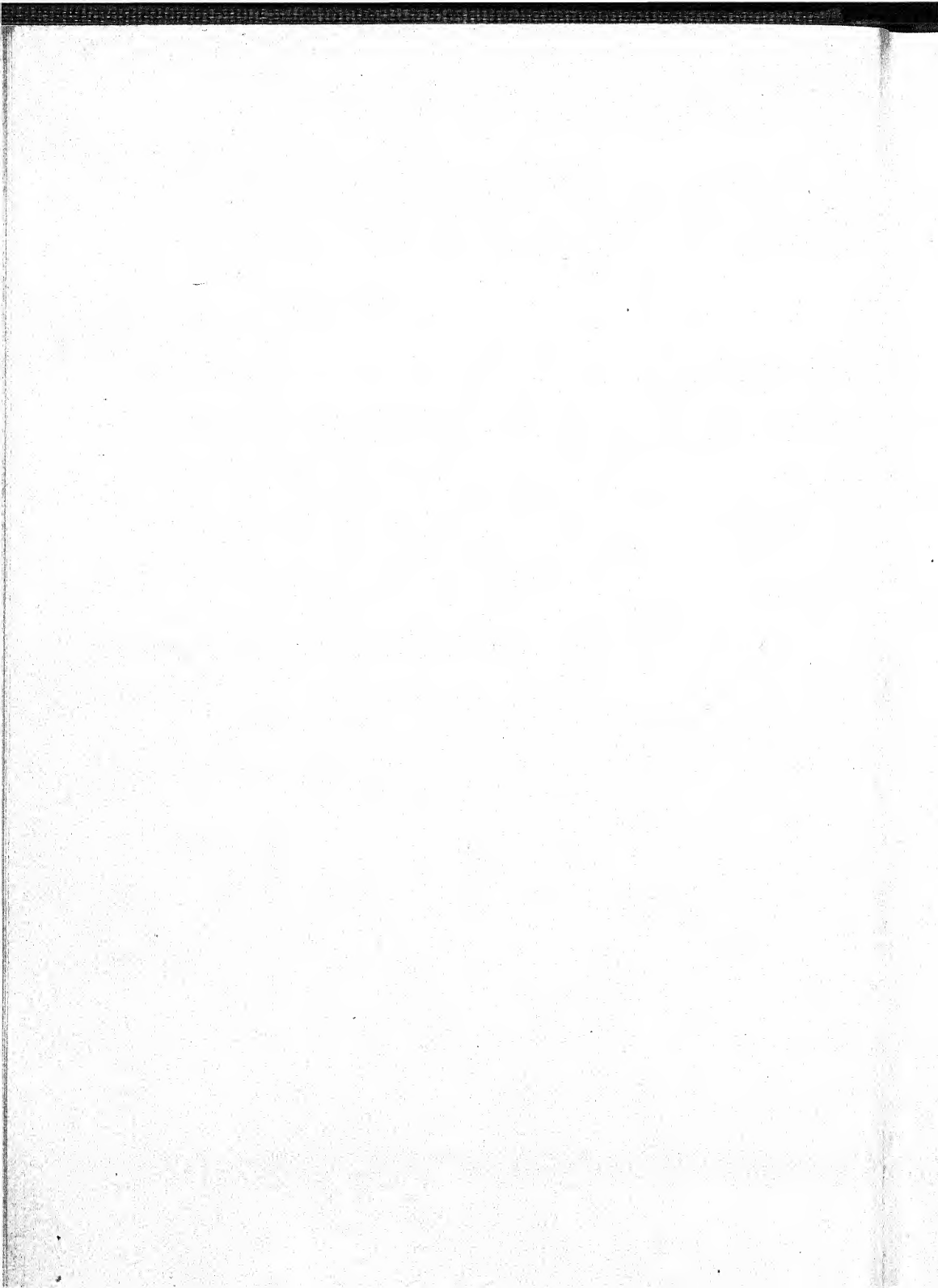
It is obvious that the biotin studied by the American workers could not be degraded to a sulfocaproic acid of this structure. Substantiation of these results by the synthesis of α -biotin would demonstrate a truly remarkable example of structural isomerism in the field of vitamin chemistry.

Based on the marked similarity in chemical and physical properties of α - and β -biotin, and on the isolation of adipic acid by oxidative degradation of biotin, as described by the American workers (48, 50), Kögl and ten Ham (120) suggest the following structure for β -biotin from liver.



It can be seen that this structure is identical with formula III discussed in this review (page 51), and was ruled out as a possible structure for the biotin studied in this country by later work apparently not known to the European workers at the time of their publication of this structure.

118. Kögl, F., Erxleben, H., and Verbeek, J. H., *Z. physiol. Chem.* **276**, 63 (1942).
119. Kögl, F., and ten Ham, E. J., *Naturwissenschaften* **31**, 208 (1943).
120. Kögl, F., and ten Ham, E. J., *Z. physiol. Chem.* **279**, 140 (1943).
121. Kögl, F., Verbeek, J. H., Erxleben, H., and Borg, W. A. J., *Z. physiol. Chem.* **279**, 121 (1943).



The Nutritional Requirements of Primates Other than Man

By PAUL L. DAY

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I. INTRODUCTION

The most conspicuous feature of the literature of primate nutrition is its paucity. Ruch (1941), in his bibliography of primates other than man, lists 4,630 citations of papers published before 1940; of those only 45, or less than one percent, are concerned with the nutrition of primates. In contrast more than 2,500 of the cited papers deal with the anatomy of primates. In the first 24 volumes of the *Journal of Nutrition* (1928–42), 1219 research papers were published; only five of those reported studies on the nutrition of primates other than man. The literature of recent years, however, reflects an increased interest in the use of primates as experimental animals in nutrition studies; more than half of the papers on primate nutrition cited in the attached bibliography have appeared within the past eight years.

Feeling that a complete bibliography of primate nutrition would be of value to investigators starting studies on monkeys and other primates, the author at the outset hoped to be able to find and cite every published work in the field. That has been done insofar as it was possible in the limited time available. Omissions have undoubtedly been made, however, but they have been through oversight rather than intent. Something of the problem involved in scanning the literature for papers on this subject will be understood when attention is called to the fact that few research journals index the species of animal used. Ruch's bibliography (1941) furnished the citations of several papers which might otherwise have been overlooked.

The subject of energy metabolism and requirement is not included in this review. Ruch (1941) gives several references to work prior to 1940 on this

subject. Very few reports have been found upon the mineral requirements of primates. With the exception of a discussion of calcium and phosphorus intakes as they relate, directly or indirectly, to vitamin D, this review does not cover the subject of mineral requirements of primates.

The nomenclature of primates is somewhat confusing to one who has not made a careful study of the subject. Zuckerman and Fulton (1934) have published a brief and very useful list of the primates commonly used in laboratory work and their characteristic features. Miller (Hartman and Straus, 1933) has given a brief but valuable discussion of the macaques, including a key to the main groups of the genus. With very few exceptions the experimental work upon the nutrition of primates has been upon monkeys; in most cases some species of macaque has been used. The most common primate found in use as an experimental animal is the rhesus macaque (*Macaca mulatta*); throughout this review it is referred to as the rhesus monkey. The word macaque is likewise used as the common name for the genus *Macaca*. In all other cases this reviewer has used the term employed by the original author.

II. VITAMIN A DEFICIENCY

A search of the literature has revealed seven experimental studies of vitamin A deficiency in the monkey. In six of these the rhesus monkey was used; in the seventh (Saiki, 1929) the species was not named. A total of 58 monkeys were given vitamin A-deficient diets in these experiments and 28 monkeys were used as positive controls. All were growing, immature animals; some were juvenile and some adolescent. The weights were between 550 and 3,600 grams. The most pertinent data from these experiments are tabulated in Table I. It will be seen from the table that only three laboratories observed any eye signs: Saiki (1929) observed xerophthalmia in two out of three monkeys, on the 30th and 47th days respectively. It may be significant that the monkeys he used were quite small. Also, since he does not name the species they may not have been rhesus monkeys, which was the species used by all the other investigators. One out of Hetler's 19 monkeys developed xerophthalmia and a perforating corneal ulcer. Verder and Petran saw no xerophthalmia, but two out of their five "developed eye symptoms not unlike nightblindness in man when placed in bright sunlight."

Harden and Zilva (1919a) and Tilden and Miller (1930) each observed one case of edema; Turner and Loew reported cessation of menstruation in their female monkeys; Verder and Petran reported data showing a slight increase in the number of leukocytes as compared to their normal animals; and Topping and Fraser found a mild gingivitis in one of their four vitamin A-deficient animals.

The most consistent deficiency sign observed was diarrhea; five out of the

seven reports refer to diarrhea in one or more of their animals, and three of the reports indicate that all or nearly all of the animals involved died with an accompanying diarrhea. A total of 32 out of 58 monkeys exhibited diarrhea. It thus appears evident that diarrhea, and not xerophthalmia, was the most consistently observed manifestation of vitamin A deficiency in the monkey.

Although the weight of evidence indicates that vitamin A deficiency, *per se*, causes diarrhea in the monkey, that evidence might well be interpreted with some caution. Diarrhea is the most obvious manifestation of a deficiency of one of the unidentified B vitamins (vitamin M) in the monkey (see section on Nutritional Cytopenia). Several of the diets used in the experiments cited in Table I contained amounts of yeast so low as to be only slightly above or possibly below the protective level of vitamin M. The Saiki diet (1929) contained 2% yeast. Since a small monkey will eat only about 75 grams of diet (dry weight basis) daily, monkeys receiving this diet would get less than 2 grams of yeast daily. In our experience 2.5 grams of brewer's yeast daily proved inadequate to protect a 2-3 kg. monkey from the cytopenia and diarrhea of "vitamin M" deficiency (Monkey No. 24, Langston, *et al.*, 1938) but 5 grams proved adequate. Saiki's experiments, however, were probably not continued long enough for nutritional cytopenia to develop.

Turner and Loew started their monkeys on the McCarrison diet (1920) which was deficient not only in the vitamins A and C, but was also deficient in the B vitamins, including "vitamin M." After the 80th day orange juice was added to the diet; on the 110th day the diet was changed to the Saiki diet which contains some, but probably not enough, "vitamin M." The monkeys exhibited a severe diarrhea but none of the classic signs of vitamin A deficiency.

Egg white furnished the B vitamins in the Hetler diet. As the "vitamin M" content of egg white has not been established, it is impossible to evaluate her diet in regard to that factor. However, the author makes a very interesting statement: "If the appetite was below normal or the stools were soft, 5 to 10 g. of dried yeast were given daily for a few days or until the appetite and stools were again normal." The poor appetite and soft stools suggest a mild "vitamin M" deficiency.

Verder and Petran used a diet containing a high percentage of egg white and some yeast, so it was probably adequate with regard to "vitamin M." Also, the fact that their deficient animals did not develop a leucopenia indicates that they were probably receiving adequate amounts of the factor necessary to prevent nutritional cytopenia. Nevertheless, all of their monkeys developed severe diarrhea. This would indicate that vitamin A deficiency, *per se*, produces diarrhea in the monkey.

Summary. Seven experimental studies on vitamin A deficiency in the

monkey from as many different laboratories are reported in the literature. In these experiments a total of 58 monkeys (mostly rhesus) were used. Only five showed eye signs: three showed xerophthalmia and two others

TABLE I
Vitamin A Deficiency in the Monkey
Summary of significant findings from various laboratories

Investigators	No. of Monkeys		Weight of Animals	No. showing the eye signs indicated	No. showing diarrhea	Other signs
	Normal controls	Deficient				
Harden and Zilva (1919a)		3	kg. 2.5-3.3	0	1	1—edema
Saiki (1929)	1	2	0.66-1.0	2—xerophthalmia on 30th and 47th days		diminished secretion of bile
Tilden and Miller (1930)	6	11	2.0-3.2	0	10	1—edema
Turner and Loew (1932)	8	14	1.5-3.0	0	severe diarrhea in all monkeys	cessation of menstruation
Hetler (1934)		19	1.0-2.0	1—xerophthalmia, corneal ulcer	2	
Verder and Petran (1937)	2	5	1.9-3.6	2—"night-blindness"	5—severe diarrhea in 8-24 weeks	slight increase in leukocytes of blood
Grinker and Kandel (1933)						
Fraser and Toping (1942)	11	4				1—gingivitis
Tomlinson (1942)						
Totals	28	58		3—xerophthalmia 2—"night-blindness"	32	2—edema

exhibited signs which were interpreted as nightblindness. Diarrhea was the most consistent deficiency manifestation; it was observed in 32 out of 58 monkeys. Other deficiency manifestations reported were: edema, cessa-

tion of menstruation, diminished secretion of bile, slight increase in white blood cells, and gingivitis. It is possible that a deficiency of other nutritional factors complicated some of the experiments. No data on the quantitative requirement of the monkey for vitamin A are available. No experiments on vitamin A deficiency in other genera of the primate order have been found in the literature.

III. RICKETS, OSTEOMALACIA, CALCIUM DEFICIENCY, AND HYPERVITAMINOSIS D IN MONKEYS

Spontaneous Rickets and Osteomalacia in Primates. Fox (1923) reported the occurrence of both rickets and osteomalacia in the primate colony of the Philadelphia Zoological Garden. He stated that a progressive type of osteomalacia occurred very frequently among the *Cebidae* (New World monkeys). On the other hand "Rickets occurred frequently in the omnivorous macaques which, however, did not show osteomalacia, although they belong to the same dietary group as the *Cebidae*. The reason they did not suffer the latter disease while adult but had rachitic young is probably due to the fact that this monkey group, which breeds best in our Garden, receives in addition to the diet given to the *Cebidae* one raw egg. . . . A few macaques dying during the delivery of young showed slight osteomalacic changes in the pelvis." It should be noted that his book was written before the etiology of rickets was well established. He points out that the muscular weakness found in osteomalacia has given rise to the term "cage paralysis," but that this term is also applied by keepers and dealers to another condition. He states: "Our Primate collection has suffered considerably with osteomalacia . . . however, this Garden is not alone in this experience."

Hottinger and Nohlen (1932) reported spontaneous rickets in three capuchin monkeys (New World, *Cebus*) in the Düsseldorf Zoo. One was diagnosed at autopsy only; the other two were diagnosed by X-ray, blood examination, and physical findings. Cure was effected slowly by the administration of massive doses of irradiated ergosterol (12 mg. 1 per cent vigantol solution daily in one case; 20 mg. daily in the other). In one animal before treatment the serum calcium was 9.6 mg. and the phosphorus 4.4 mg. per 100 ml. After treatment the calcium was 8.8 mg. and the phosphorus 7.6 mg. per 100 ml. serum. They considered the disease analogous to human rickets. No evidence of hypervitaminosis was found at autopsy 4 and 6 months later, respectively.

Experimental Rickets in Monkeys. Topping and Fraser (1939), in their study of mouth lesions in monkeys associated with dietary deficiencies, placed four monkeys on a diet deficient in vitamin D. The diet was presumably adequate with regard to calcium and phosphorus. The animals

survived for 50, 63, 101, and 127 days. At autopsy perforation of the gut was found in one, and tuberculosis in the other three monkeys. Except for information on mouth lesions the authors make no statements regarding the clinical course of the animals during the experiment.

Gerstenberger (1938, a, b, c, d) made the most careful study of experimental rickets in the monkey that I have found in the literature. It is unfortunate that he has not reported his experiments in more detail. However, by piecing together his several brief reports it is possible to get a fair idea of the nature of rickets in the rhesus monkey.

Two diets were used; one was a low-calcium diet with a Ca:P ratio of 0.49. The other was a higher calcium diet with a Ca:P ratio of 1.3. The amount of phosphorus was the same in both diets—approximately 2.56 g. weekly. The weekly intake of calcium was about 1.26 g. in the low calcium diet, and 3.08 g. in the higher calcium diet.

Large groups of juvenile monkeys, weighing 0.8 to 2.4 kg., were given these diets. With the exception of one animal all the animals in both groups developed the low phosphorus, high calcium type of rickets, which was characterized by essentially normal blood calcium levels and decidedly abnormal, low phosphorus levels. "In other words, the monkeys whose exposure to actinic rays was prevented acquired the type of rickets most frequently seen in the full term human infant." The average calcium level of the serum was 9.52 (range, 8.7–11.1) mg. per 100 ml. The average phosphorus level of the serum was 2.07 (range, 1.1–2.9) mg. per 100 ml. Exposure to ultraviolet irradiation or feeding of cod liver oil or viosterol resulted in healing of the rickets, usually by the 14th day. The healing of the rickets by exposure of monkeys to ultraviolet light was accompanied by spasmophilic signs in several of the monkeys on the low calcium diet; this was not observed in monkeys on the higher calcium diet. Various phosphate solutions given parenterally brought about healing, as shown by roentgenogram, and usually resulted in a return to normal of the blood level of calcium and phosphorus. Calcium lactate, however, was without effect. "It is suggested, therefore, that the term phosphatization rather than calcification might better be used in connection with the healing process of this form of rickets." In one monkey the rickets was healed by a single dose of 0.62 g. cod liver oil containing about 160 I.U. of vitamin D.

It is thus evident that the young rhesus monkey is quite susceptible to rickets in the absence of some source of vitamin D, seemingly without regard to the Ca:P ratio. This is in marked contrast with the rat, which develops rickets readily only when there is a deficiency of vitamin D superimposed on an unsatisfactory Ca:P ratio.

Chronic Calcium Deficiency in the Rhesus Monkey. Fraser (1942) fed four rhesus monkeys, weighing 2.53 to 3.47 kg., a diet qualitatively and

quantitatively adequate with regard to calories, protein, vitamins, and all minerals except calcium. Four other animals were given the same diet, but deficient in ascorbic acid as well as calcium. Controls were given the same diet with adequate calcium and ascorbic acid.

The calcium content of the deficient diet was 10.2 mg. per 100 g. diet (dry weight); the phosphorus content was 380 mg. per 100 g. The normal control diet contained 190 mg. of calcium per 100 grams and phosphorus as above. Although Fraser does not report food intake records, from our knowledge of the dietary requirement of the monkey it can be estimated that the two larger of the four in the calcium-deficient group would have eaten about 100 grams of the diet daily. This would make the daily intake for the deficient monkeys about 11 mg. of calcium and 380 mg. of phosphorus; for the normal controls the daily intake would be about 190 mg. of calcium and 380 mg. of phosphorus. The daily intake of Gerstenberger's (1938) monkeys on his low calcium rachitic diet was 180 mg. of calcium and 366 mg. of phosphorus daily—almost identical with Fraser's normal control diet. It must be remembered, of course, that Fraser's diets were vitamin D-adequate, and Gerstenberger's diets were vitamin D-deficient.

Fraser's calcium-deficient monkeys exhibited no conspicuous clinical signs for 11 months, and they made fair gains in weights. One showed a moderate decline in weight after ten months, but one was on diet 488 days before declining in weight. Toward the end of the experiment they showed symptoms of irritability, anorexia, and weakness of the hind legs. One would scream and shriek when being caught in the net and would "tremble all over" while receiving an intramuscular injection of vitamin C. There was considerable muscular atrophy and loss of tone. Analysis of bones showed a marked reduction in the calcium content. There was also a distinct reduction in the content of calcium in blood serum; one value of 5.0 mg. per 100 ml. serum was found. There was no distinct tetany at any time, however.

Fraser's description of the marked muscular weakness in chronic calcium deficiency is strongly suggestive of the "cage paralysis" (osteomalacia) in primates described by Fox (1923).

Hypervitaminosis D in the Monkey. The rhesus monkey appears to, be rather resistant to overdosage of vitamin D.

Hess and Lewis (1928) found latent tetany, manifested by a serum calcium content of about 6 mg. per 100 ml., in a monkey maintained on a low calcium diet. It was found possible to raise the serum calcium level to about 11 mg. by means of large amounts of irradiated ergosterol by mouth. However, after removal of the parathyroid glands, the calcium could not be raised much above 7 mg. per 100 ml. Hess, Weinstock, and Rivkin carried out "numerous" experiments on monkeys and dogs, and they stated that

giving irradiated ergosterol would produce hypercalcemia; levels of 13-16 mg. per 100 ml. were produced in this way. Irradiated ergosterol was found to increase also the inorganic phosphorus of the serum, frequently to levels of 12 to 14 mg. per 100 ml. of serum. The details of their experiments were not given.

Levaditi and Po (1930) gave irradiated ergosterol to two monkeys, one a 1,250 gram *Cercopithecus callitrix* and the other a 750 gram rhesus. The dosage was "30 to 60 times greater than that used therapeutically in humans." At autopsy no lesions were observed in the *Cercopithecus*, and only very slight calcification was seen in the kidney of the rhesus monkey.

Cowdry and Scott (1935, 1936) gave irradiated ergosterol to 19 juvenile and adolescent rhesus monkeys and to two New World Monkeys (*Cebus fatuellus*). Twenty-four untreated rhesus monkeys were used as controls. The average serum calcium in the rhesus monkeys was 11.6 mg. in males and 11.0 mg. per 100 ml. in females. The average serum phosphorus was 5.98 mg. in males and 6.0 mg. in females. Irradiated ergosterol was given in several ways: (a) two monkeys received single large doses of about 5,000,000 units of vitamin D; (b) three monkeys received double doses, each dose as above; (c) one monkey received three doses, containing a total of nearly 30,000,000 units of vitamin D; (d) seven monkeys received repeated doses over long periods; and (e) three monkeys received doses heavy enough to increase the blood calcium. One in this latter group received a total of approximately 122,000,000 units of vitamin D divided into 28 doses.

When it is remembered that the daily protective dose for a normal infant is approximately 1,000 units, and the usual daily curative dose for cases of infantile rickets is 3,000 to 4,000 units daily (New and Nonofficial Remedies, 1942), it is evident that the doses given by Cowdry and Scott were rather large.

The average level of serum calcium remained about the same during treatment. However, in the three monkeys receiving massive doses elevated calcium levels were found; one value of 20.3 mg. per 100 ml. was recorded. The serum phosphorus in the treated monkeys increased to an average of 7.75 mg. in the males and to 7.44 mg. in the females. Histologic manifestations of vitamin D overdosage described in the literature for other species were *not* found in the rhesus monkeys, although certain mild tissue changes previously unreported were found. The response of the two mature *Cebus fatuellus* monkeys to irradiated ergosterol was so different from the response of the rhesus monkeys that Cowdry and Scott warned against applying to children their conclusions drawn from the behavior of rhesus monkeys.

Summary. Both rickets and osteomalacia are reported to occur spontaneously in captive primates. Rickets has also been produced experimentally in young rhesus monkeys deprived of vitamin D and protected from actinic rays.

Under such conditions young of the species develop a low phosphorus, high calcium type of rickets. Prolonged calcium deficiency in monkeys given an otherwise adequate diet has produced a syndrome characterized by irritability, anorexia, weakness, and muscular atrophy; analysis of bones revealed a marked reduction in calcium content. The rhesus monkey appears to be rather resistant to overdosage of vitamin D.

The vitamin D requirements of primates other than man have not been established, and few studies have been made upon the calcium and phosphorus requirements of those species.

IV. SCURVY—ASCORBIC ACID REQUIREMENT OF THE MONKEY

Experimental and Spontaneous Scurvy in Monkeys. Scurvy was the first deficiency disease to be produced experimentally in the monkey, and the ascorbic acid requirement of this species has been established with a higher degree of accuracy than has its requirement for any other substance. In fact, the ascorbic acid requirement of the monkey is the only nutritional requirement which is known to any fair degree of certainty.

Hart reported in 1912 the experimental production of scurvy in the monkey by feeding a diet of condensed milk, and he showed the identity of this experimental condition with human infantile scurvy. His reports give the clinical signs of monkey scurvy, and also detailed post-mortem studies. The monkeys exhibited a general listlessness and loss of vigor. The first pathological sign was hemorrhage of the gums. Subperiosteal hemorrhages were also seen, in some cases consisting of large effusions. Hemorrhage into the orbit leading to exophthalmos was observed, as was hemorrhage into and resulting discoloration of the upper lid. Swelling of the epiphyseal ends of the long bones was frequently found, and fractures were common, especially of the lower part of the femur and the upper part of the humerus. Bloody extravasations were found in the meninges, lungs, kidneys, and intestinal mucosa. Severe changes were seen in the bones of the jaw and skull. Hess in 1920 reviewed the gross and microscopic findings in experimental scurvy in the guinea pig and the monkey. He pointed out certain features of monkey scurvy that are not consistently found in guinea pig scurvy—hemorrhage of the gums, large subperiosteal hemorrhage and effusions, and ocular hemorrhage and exophthalmos—and he stated that these distinctions render scurvy in monkeys and infants practically identical. In most respects, however, monkey scurvy closely resembles guinea pig scurvy.

Talbot, Dodd, and Peterson (1913) repeated Hart's experiments, using two rhesus monkeys. One received a normal diet and the other was given a scorbutic diet consisting of condensed milk. The latter animal developed listlessness, anemia, purplish-red spongy gums, but no tenderness around

the bones nor subperiosteal hemorrhage. The animal died three months after the condensed milk was started and nine days after the redness of gums was first observed. The autopsy findings in the bones were not characteristic of scurvy.

Harden and Zilva (1919) produced scurvy in three rhesus monkeys, using a diet consisting of steamed and autoclaved foods (steamed wheat germ; autoclaved bread, monkey nuts, milk, and rice). One of the animals also received 150 cc. of fresh beer daily. The clinical findings were: diminution of activity, weakness of joints, bleeding, spongy gums, loose teeth, loss of appetite, and muscular tenderness. Post mortem findings included: subcutaneous and intramuscular hemorrhages; subperiosteal hemorrhages of the skull, femur, tibia, ulna, and abdominal wall; "rosary" swellings and subperiosteal hemorrhages of the ribs. One survived 134 days and the second died on the 121st day following a severe diarrhea. On the 99th day the condition of the third monkey was critical but it was recovered by feeding lemon juice from which the citric acid had been removed.

Howe (1923, 1924) developed scurvy in a large number of rhesus monkeys, using a diet of the following percentage composition: wheat, 35; soy beans, 25; rolled oats, 22.5; powdered milk, 10; butter, 5; calcium carbonate, 1.5; and sodium chloride, 1. Several modifications of this diet were also used; in one diet the calcium carbonate was omitted. Although Howe was primarily interested in the teeth and related structures, he briefly reported the following general scorbutic findings: edema and hemorrhage of the tissues around the eyes, exophthalmos, swollen and painful joints, scorbutic rosary of the ribs, and decalcification and fracture of the bones. Marked changes in the teeth and supporting tissues were found by gross examination, X-ray examination, and post-mortem studies. "Every stage of peridental trouble, from slight gingival redness to the complete destruction of the supporting tissues has developed on these deficient diets." Tartar deposits, loosening of the teeth, hypertrophy or sloughing and necrosis of peridental tissue, and decalcification of the jaw bone were observed. Changes in the structure of the teeth were observed, and cavities were found in the teeth of many of the monkeys. The author was not fully convinced, however, that a deficiency of vitamin C was the sole cause of the caries.

Howitt (1931) reported a spontaneous outbreak of scurvy in a shipment of 39 rhesus monkeys shortly after their arrival from India; this appears to be the first record of spontaneous scurvy in monkeys. The diet enroute had been composed largely of grains and seeds, with little or no green vegetables. The most consistent manifestation of scurvy was bleeding gums; other signs recorded were: cushion-like cranial swelling, swollen eyelids, lameness, swollen joints, cushion-like swellings of ear, and general weakness.

A change of diet to one rich in ascorbic acid (orange, raw cabbage, bananas) produced marked improvement within a few weeks with subsequent complete recovery. In those with advanced scurvy which were put to death or which died within the first few days, post-mortem findings were characteristic of scurvy.

Miura and Okabe (1933) produced scurvy in a "Formosan long tail female monkey (*Macacus cylopsis swinhoi*)," about two years of age and weighing approximately 4 kg., by feeding a diet of autoclaved milk and autoclaved white bread, supplemented with salt mixture, B vitamin solution, and vitamin A. Severe scurvy developed in about three months. In addition to the characteristic loss of vigor, hemorrhagic gums, and pain, the monkey developed a bloody diarrhea. They reported that the daily feeding of 150 cc. of fresh commercially sterilized milk failed to effect a cure, but that the daily administration of an infusion prepared from 0.5 gram of green tea resulted in complete recovery in one month.

Topping and Fraser (1939) and Tomlinson (1939) reported gross and microscopical findings in the mouths of four rhesus monkeys restricted to a diet deficient in ascorbic acid. The diet used was adequate with regard to all other nutritional essentials since it contained casein, salt mixture, about 10% dried brewer's yeast, and was supplemented with carotene and crystalline vitamin D. Early mouth lesions (redness and slight hemorrhage of gums, gingivitis) were first noted in 14, 15, 15, and 43 days in the four monkeys, respectively. In two cases the gum lesions progressed to necrosis. Smears from the gums showed an increase in fusiform and spiral organisms. At death the mouths exhibited the following lesions: soft tartar, inflammation, localized recession of gums, necrosis of interdental papillae, and necrotic gingivitis. On the basis of autopsy findings, scurvy was given as the cause of death in all four cases, although two were complicated with tuberculosis. The survival periods were 75, 75, 111, and 120 days (average, 95 days).

Sabin (1939) recorded some interesting observations on a series of monkeys receiving a scorbutic diet; 13 out of 25 monkeys succumbed to acute infections (pneumonia, enterocolitis, and others) after they had been on diet between 9 and 18 days, and before evidences of ascorbic acid deficiency appeared. Control monkeys receiving adequate ascorbic acid remained well.

Chapman and Harris (1941) fed five rhesus monkeys a scorbutic diet identical to the one used by Topping and Fraser, and observed similar mouth lesions. The average survival of their five monkeys was 89 days. Autopsy findings indicated scurvy as the cause of death in every case, but one was complicated with bacillary dysentery.

One of the monkeys (No. 47) of Day, Langston, Darby, Wahlin, and Mims (1940) was given a diet deficient in both "vitamin M" and ascorbic

acid. By the 94th day the monkey exhibited most of the classic signs of nutritional cytopenia: a profound anemia and leucopenia, and diarrhea. Some edema of the face was evident, but the condition of the gums remained good. The feeding of liver extract was followed by a reticulocyte crisis and the prompt return of all white blood cells and erythrocytes toward normal levels. The diarrhea disappeared and the edema subsided. On the 112th day, however, the gums appeared spongy and the animal gave evidence of muscular tenderness upon being handled. These signs were interpreted as indicating scurvy, and thereafter 50 mg. of ascorbic acid daily was given the animal. This supplement was followed by the disappearance of the gum changes and the muscular tenderness. The interesting feature of this experiment is that nutritional cytopenia was developed, and cured, before scurvy was evident.

Fraser (1942), Fraser and Topping (1942), and Tomlinson (1942) reinvestigated certain phases of the problem of ascorbic acid deficiency in the monkey, with and without a concurrent calcium deficiency. The diet used was presumably adequate with regard to all other dietary essentials. Three of the eight monkeys showed a marked reduction in blood hemoglobin concentration, but none showed a reduction in erythrocytes. The average white blood cell count was reduced from 16,600 to 12,800 per cu.mm., but this latter is still well within the normal range for the rhesus monkey. The younger ascorbic acid-deficient monkeys showed a reduction in the percentage calcium content of the bone, but this reduction was not as marked as that observed in calcium-deficient monkeys or in monkeys with a concurrent ascorbic acid and calcium deficiency. The scorbutic monkeys showed no reduction in blood serum calcium, nor in total serum proteins, but they did exhibit a reversal of the serum albumin:globulin ratio. "The conspicuous symptoms of chronic vitamin C depletion observed in the monkeys of this experiment are: Anorexia, loss of weight, loss of hair, hemorrhage of the gingiva and other tissues, inability to walk because of extensive joint hemorrhage, and finally ankylosis of knee joints." In the cases where gum lesions had advanced only to the stage of inflammation and hemorrhage, treatment with ascorbic acid arrested the condition promptly and restored normal tissue. Where, however, the scorbutic lesions had advanced to the necrotic stage, many changes occurred in the gingiva and peridontal tissue which did not respond to therapy and were considered to be irreversible in that respect.

Quantitative Ascorbic Acid Requirement. Harden and Zilva (1920) attempted to establish the antiscorbutic requirement of the monkey. Five rhesus monkeys, weighing between 2160 and 3300 grams, were given a scorbutic diet, supplemented with 5 cc., 2 cc., 1 cc., 0.75 cc., and 0.5 cc. of orange juice daily, respectively. The monkeys receiving 5 cc. and 2 cc. of

orange juice were fully protected from scurvy; the latter experiment lasted for one year, and the monkey increased in weight from 3300 to 4400 grams. The monkey which received 1 cc. of orange juice exhibited spongy gums and loose teeth after three months on the diet. The monkeys which received 0.75 and 0.5 cc. of orange juice daily developed severe scurvy, surviving 116 and 161 days, respectively. The authors concluded that the minimum daily dose of an antiscorbutic for the monkey lay between 1 and 2 cc. of fresh orange juice daily. If we assume that 2 cc. of fresh orange juice was their minimum protective dose, and if we further assume that each cubic centimeter of their orange juice contained 0.5 mg. of ascorbic acid (Sherman, 1941; Daniel and Munsell, 1937) we may interpret their results as indicating that the minimum daily dose of ascorbic acid required to prevent scurvy in a 2-4 kg. rhesus monkey is approximately 1 mg.

Langston, Darby, Shukers, and Day (1938) maintained three rhesus monkeys (Nos. 24, 27, 29) for extended periods on a diet of refined food-stuffs supplemented with 4 grams of orange daily. Although these experiments were designed for other purposes, they are of value in estimating the ascorbic acid requirement of this species. The B vitamins were supplied by dried brewers yeast in two cases and by extralin (a liver-stomach preparation) in the third. Except for a paralysis of unknown etiology in two of the animals, the monkeys were still normal at the end of 429, 596, and 572 days, respectively. There was no evidence of scurvy at any time. One (No. 27) increased in weight from 1760 to 3427 grams; the other larger monkeys made somewhat smaller gains. Dentition progressed normally. If we assume that each gram of orange contained 0.5 mg. of ascorbic acid, it is evident from these experiments that 2 mg. of ascorbic acid daily was fully adequate to protect juvenile rhesus monkeys from scurvy and to promote growth and development for extended periods.

Although the experiments of Fraser (1942) were not continued for as long a time as the foregoing, they are more quantitative inasmuch as he used pure ascorbic acid as the vitamin C supplement. Eight of his monkeys were given an ascorbic acid-deficient diet supplemented with 3 mg. of ascorbic acid daily for 81 days and 1.29 mg. daily (9 mg. each week) for the subsequent 127 days. Thus over a period of 208 days the monkeys each received an average daily intake of 1.96 mg. ascorbic acid. Seven of the monkeys (weighing between 2.50 and 3.64 kg. at the start of the experiment) were fully protected from scurvy during the period of vitamin supplement. The monkeys then developed scurvy in 21, 21, 21, 29, 29, 44, and 44 days after withdrawal of ascorbic acid. The eighth monkey, which weighed 5.2 kg. at the beginning of the experiment, began to show evidence of scurvy after one month on a vitamin C intake of 3 mg. per day. It continued to gain in weight, however, throughout the period of low vitamin intake,

reaching a maximum of 7.64 kg. It is thus evident that an average daily intake of 1.96 mg. ascorbic acid was adequate to protect rhesus monkeys, weighing 3.64 kg. or less, from scurvy for a period of 208 days. For a larger monkey, weighing 5.2 kg. at the start of experiment, 3 mg. per day was insufficient.

Greenberg, Rinehart, and Phatak (1936) found the plasma ascorbic acid levels of a monkey receiving about 3 mg. of ascorbic acid daily (6 cc. orange juice) to be essentially the same as the plasma ascorbic acid levels of a monkey receiving no vitamin C supplement. These data would indicate that 3 mg. of ascorbic acid daily was near the minimum protective dose for that particular animal (No. 1001) which weighed 3.3 kg.

In a personal communication, Dr. Rinehart interprets their data as indicating that the requirement of a 3.3 to 4.5 kg. monkey is between 1.5 and 3 mg. ascorbic acid daily. "If the requirement lay between 1.5 and 3 mg. daily, this would be about 0.5 to 0.7 mg. per kilogram, which is rather close on a weight basis to the requirement of man."

From these experiments we may tentatively set the antiscorbutic requirement of a rhesus monkey, weighing less than 4 kg., at 2 mg. or less per day. This should be taken as the amount of ascorbic acid necessary to prevent scurvy; the intake for optimum nutrition is probably much higher.

Ascorbic Acid Content of Monkey Tissues. Greenberg, Rinehart, and Phatak (1936) determined the reduced ascorbic acid content of blood of four rhesus monkeys over a period of four months, and under several conditions of vitamin C intake. One monkey on a sub-optimal intake of vitamin C (approximately 6 cc. of orange juice daily, which would contain about 3 mg. ascorbic acid) showed blood ascorbic levels essentially the same as those of a monkey receiving no ascorbic acid supplement: between 0.1 and 0.2 mg. ascorbic acid per 100 ml. plasma. Two monkeys receiving a liberal intake of vitamin C (half an orange daily, each) exhibited higher, but variable, plasma ascorbic acid levels: values as low as 0.3 mg. and as high as 1.1 mg. were found but most of them were between 0.3 and 0.8 mg. per 100 ml. of plasma.¹

Kasahara and Gammo (1937) found the average ascorbic acid content of the cerebrospinal fluid of 6 monkeys to be 2.3 mg. per 100 ml. with wide individual variation. The ascorbic acid content of the cerebrospinal fluid of 7 monkeys was determined after the animals had been on a scorbutic diet two weeks but before symptoms of scurvy appeared; the ascorbic acid level of the fluid decreased to half its normal value and continued to decrease rapidly. Subcutaneous injection of 200 mg. of ascorbic acid daily restored

¹ Dr. Rinehart made the following statement in a personal communication: "To get true blood vitamin C concentration from my charts you should subtract 0.12 mg. because the figures charted were not corrected for the blank values."

the vitamin C content of the cerebrospinal fluid to normal in two days, but continued injection of ascorbic acid for 32 to 41 days did not increase the vitamin C content above twice the normal level.

Jungeblut and Feiner (1937) determined the ascorbic acid content of brain, cord, suprarenal, and other tissues of groups of monkeys, using the dye titration method. Four groups of monkeys were used: (1) normal monkeys, with no parenteral ascorbic acid; (2) C-treated normal monkeys, which were normal monkeys receiving 5 or 25 mg. of ascorbic acid subcutaneously each day for a period of two weeks; (3) poliomyelitic control monkeys, receiving no added ascorbic acid; and (4) C-treated poliomyelitis-infected monkeys, which received 5 to 100 mg. of ascorbic acid daily for two weeks. The content of ascorbic acid in brain, cord, and suprarenal was higher in the C-treated animals (groups 2 and 4) than in the monkeys which did not receive ascorbic acid subcutaneously (Groups 1 and 3). The average amounts of ascorbic acid in the tissues of the normal untreated monkeys were: brain, 0.08; cord, 0.07; and suprarenal, 0.43 mg. per g. of tissue. The authors compare the ascorbic acid content of these tissues of monkeys with those of the corresponding tissues of the rat, rabbit, cat, dog, guinea pig, and man. They point out that the levels of ascorbic acid in the tissues of species capable of synthesizing ascorbic acid (rat, rabbit, cat, and dog) are higher than the content in the corresponding tissues of animals which depend upon a dietary intake of vitamin C (guinea pig, monkey, and man).

Ascorbic Acid and Experimental Monkey Poliomyelitis. Jungeblut showed (1935, 1939) that both the Aycock strain and the RMV strain of poliomyelitis could be rendered non-infectious when treated with ascorbic acid *in vitro*. Furthermore, the treatment of monkeys with ascorbic acid after the intracerebral administration of the Aycock strain of the virus afforded definite protection against paralysis (Jungeblut, 1937, 1937a). On the other hand, Sabin (1939) found that ascorbic acid treatment gave no protection against paralysis in monkeys which had been given the RMV strain of poliomyelitis virus intranasally. In a later series of experiments, Jungeblut (1939) used the RMV strain of virus, given by the nasal route, and found partial protection by the use of ascorbic acid. In view of the apparent discrepancy between the work of Jungeblut and of Sabin it is difficult to evaluate the effectiveness of ascorbic acid in the protection of monkeys from the virus of poliomyelitis.

Summary. Numerous data indicate that the monkey is readily susceptible to scurvy. The time required for the development of scurvy and the survival periods are quite variable, and they probably depend in part upon the age and weight of the animal and upon the diet used. Clinical observations, post-mortem findings, and histopathological studies

indicate that monkey scurvy is identical with human scurvy. All the published experiments have been upon macaques and all of those except one were upon rhesus monkeys (*Macaca muatta*). No record has been found of experiments upon other non-human primates, so it is not known whether other genera in the order are susceptible to scurvy.

The minimum ascorbic acid requirement of a rhesus monkey weighing 2 to 4 kg. is 2 mg. or less a day. The ascorbic acid requirement for optimum nutrition has not been established. Some data are available on the blood and tissue concentration of ascorbic acid in this species. It has been reported that treatment with ascorbic acid increases the resistance of the monkey to poliomyelitis virus, but the validity of those findings has been questioned.

V. BERIBERI IN THE MONKEY

A search of the literature reveals that a number of attempts have been made to produce beriberi in the monkey. Although most of those experiments fall far short of what we would consider well-controlled animal experiments today, they do yield some valuable information.

Wright (1903) incarcerated monkeys ("*M. cynomolgus*" and "*M. nemestrinus*") in certain cells of the Kwala Lumpor (Malay) jail, in which prisoners had developed beriberi. His monkeys exhibited signs which he considered to be convincing evidence of beriberi: anorexia, changes in knee-jerk, wrist-drop and foot-drop, and paralysis. He believed that the monkeys contracted the disease by contact with pathogenic organisms from the cases of human beriberi previously housed in the cells of the jail, and he concluded that beriberi is an acute infectious disease. His monkeys were given a diet of pineapple, banana, and sugar cane. Pineapple and banana are at least fair sources of thiamin (Sherman, 1941). It would be fruitless to speculate on the identity of the disease which his monkeys developed, but it seems highly improbable that they suffered from thiamin deficiency.

Schaumann (1910) briefly reported the occurrence of what he believed to be beriberi in one monkey.

Shiga and Kusama (1911) fed two Japanese monkeys ("*Macacus cynomolgus*") a diet of cooked rice. One died of tuberculosis, but the other developed a syndrome characterized by loss of appetite, declining weight, progressive paralysis of hind legs, disappearance of knee reflexes, edema, and dilatation of the heart. The pulse rate decreased from a normal of about 250 to 180 per minute. From physical findings and from histopathological studies they concluded that the disease was very similar to human beriberi.

McCarrison (1919, 1919a) fed numerous monkeys on various diets

deficient in the B vitamins, but from his description of the deficiency manifestation it appears most likely that he was dealing not with a deficiency of thiamin, but with a deficiency of another member of the vitamin B complex. His work is discussed in the section on Nutritional Cytopenia.

Wechsler, Jervis, and Potts (1936) studied the effect upon rhesus monkeys of (a) acute and chronic alcohol intoxication, (b) "vitamin B" deficiency, and (c) "vitamin B" deficiency associated with alcoholic intoxication. There was no clear-cut evidence of polyneuritis in any of the groups. Certain of the animals in the deficient group and in the deficient group receiving alcohol developed severe diarrhea, strongly suggestive of nutritional cytopenia.

Leblond and Chaulin-Servinière (1942) reported 20 cases of spontaneous beriberi in the colony of monkeys ("*Macacus sylvanus*") in the Pasteur Institute of Morocco, following a change in diet. Before February, 1940, the diet of the monkeys consisted largely of acorns, supplemented with small amounts of stale bread, carrots, cabbage, and sometimes sunflower seed. Acorns became unavailable at that time, and the diet was changed so as to consist of noodles, semoule, chick peas, broad beans, carrot, turnip, cabbage, and white wheat bread. The investigators estimated that the average thiamin intake was about 23 micrograms daily per monkey. The first case of beriberi appeared within two months after the change in diet. The most marked clinical findings in the 20 cases were: muscular weakness and eventual paralysis of the hind legs, muscular atrophy, dyspnea, decreased pulse rate, and loss of appetite. Attempted cure by the subcutaneous administration of 5 to 50 mg. of thiamin failed in three terminal cases, but partial recovery was effected by the addition of 5 mg. daily to the drinking water of each of six animals.

The authors attempted to produce beriberi experimentally by feeding certain of their monkeys an artificial diet entirely devoid of the B vitamins. Two adolescent monkeys, which had been on the beriberi-producing stock diet but had not become severely ill with beriberi, were given this vitamin-free diet. The effect of the diet was quite rapid, resulting in a sudden and complete paralysis of all four extremities in 3 and 6 days in the two monkeys. They considered these two cases comparable to "fulminating beriberi" in humans. Attempts to produce beriberi in other monkeys by feeding them the experimental diet failed, however, as the monkeys died in 33 to 40 days with very few symptoms. The authors suggest that beriberi can only be produced in the monkey by feeding a diet partially deficient in thiamin.

It would appear from the foregoing that three laboratories have observed beriberi in monkeys. Other laboratories, however, have failed to observe beriberi when monkeys were restricted to a diet deficient in the B vitamins;

in some cases at least this failure was probably due to the early appearance of syndromes resulting from deficiencies of other essential substances. Much remains to be learned regarding the conditions under which thiamin deficiency develops in primates, the relative susceptibility of various primate species to beriberi, and the quantitative requirement of these species for thiamin.

VI. EXPERIMENTAL ATTEMPTS TO PRODUCE PELLAGRA IN MONKEYS

The literature includes a number of reports of attempts to produce pellagra experimentally in monkeys. Since many of those attempts were made before the etiology of human pellagra was understood, the early reports are of historical interest only.

Medical thinking with regard to the etiology of pellagra has passed through three periods, during each of which a different theory of the cause of the disease was more or less accepted. The etiological theories coincident with these three successive periods were: (1) the infectious theory, (2) the protein deficiency theory, and (3) the vitamin deficiency theory. In reading the literature one finds experiments on the monkey in each of these periods; experiments designed to produce, if possible, pellagra in the monkey by technics based upon the then accepted etiological theory.

To test the infectious theory, Anderson and Goldberger (1911) injected blood and spinal fluid from pellagrins into five rhesus monkeys. Lavinder (1911) inoculated nine monkeys with blood, spinal fluid, and an emulsion prepared from the spinal cord of pellagrins. Blood and spinal fluid was given intraperitoneally; the emulsion of spinal cord was given intracerebrally. None of the monkeys in these two experiments showed any changes which could be called pellagra.

On the other hand Harris (1913) produced in three monkeys a condition which he believed to be pellagra by the injection of a Berkefeld filtrate of material obtained from fatal cases of human pellagra. The filtrate was prepared from the brain and cord, skin lesions, and portions of the alimentary tract, and was injected into the monkeys intracranially, intravenously, and subcutaneously. Several months later lesions appeared on the face and hands which led the author to believe that he had transmitted pellagra from the human to the monkey. Lavinder, Francis, Grimm, and Lorenz (1914) were unable to repeat the work of Harris. They injected filtrates from brain, spinal cord, and various visceral organs into a large number of monkeys. Extracts from intestinal contents were injected intravenously, intracerebrally, and subcutaneously into rhesus monkeys, Java monkeys, and one baboon. Cerebrospinal fluid, pericardial fluid, and Berkefeld filtrates of urine and feces were injected into many monkeys. Tissues and excreta from pellagrins were also fed to a number of monkeys.

In all, 103 experiments were conducted on seventy-seven rhesus monkeys, two Java monkeys, and three baboons. In only one animal did lesions appear which were suggestive of pellagra.

Chick and Hume (1920), in planning their experiments on the monkey, were obviously influenced by the protein deficiency theory of the cause of pellagra, which had only recently been proposed by Goldberger. They produced what they believed to be symptoms closely resembling pellagra by feeding three rhesus monkeys a diet containing corn gluten as the chief protein. Other constituents of the diet were butter, banana or apple, sugar, and orange juice. Each animal received two grams of yeast daily. One animal (Lazarus) lost weight, showed mild lesions of the mouth and a severe diarrhea, and was chloroformed on the 90th day while moribund. The two other monkeys lost weight more slowly and one developed edema. Additions of lysine and tryptophan failed to alter the downward course of the animals; both were recovered by returning them to a normal mixed diet. One of the three monkeys exhibited florid skin lesions suggestive of pellagra. It is somewhat difficult to evaluate this experiment, since the monkeys were offered several foods which were not all eaten in every case. The 2 grams of yeast daily may not have provided a sufficient amount of the B vitamins; the gum lesions and diarrhea shown by Lazarus are strongly suggestive of nutritional cytopenia.

Turning to the vitamin deficiency period in the history of the etiology of pellagra, several papers have reported attempts to produce monkey pellagra. Efremov (1936) fed two rhesus monkeys on a mixed cereal diet consisting of middlings, barley, white rice, wheaten bread, sugar, and carrots. One developed a polyneuritic condition after six months and the other a pellagra-like affection after eight months. He suggested that the type of vitamin deficiency developed depended on the individual diathesis and varying conditions of the gastro-intestinal tract. As the paper is available in abstract form only, evaluating it is difficult. In any event an experiment upon two animals only cannot be considered conclusive.

Clark (1938) experimented upon two elderly female baboons at the Zoological Gardens at Cairo. They each received durra, *ad libitum*, 150-200 g. carrot, and $\frac{1}{4}$ pint of milk daily. One also received dates *ad libitum*; this animal remained in good health. The one which did not receive dates ceased menstruation, became weak, the palms of the hands became swollen, and a red rash appeared over her abdomen, around the eyes, and especially on the eyelids. Later a hemorrhagic rash appeared over most of the body, and the animal eventually succumbed in a coma. The author considered the syndrome to be pellagra.

Following the discovery that nicotinic acid could prevent and cure black-tongue in dogs and pellagra in man, attempts have been made to produce

nicotinic acid deficiency in monkeys. By feeding the Goldberger diet to monkeys, Harris (1937, 1938) produced a syndrome characterized by loss of appetite, profuse diarrhea, vomiting, anemic appearance, progressive weakness, denudation of fur, dry scaly skin, and eventual death. All of these manifestations could be prevented or cured by 7% yeast in the diet, by feeding a crude liver extract, or by replacing the maize in the Goldberger diet with wheat. In the second paper Harris referred to this syndrome as "monkey pellagra," and reported data on four monkeys in which weight responses were obtained by feeding nicotinic acid. No blood counts were reported so it is not evident whether the nicotinic acid influenced the anemia.

In this country two laboratories have reported experiments on monkeys given the Goldberger diet supplemented with ascorbic acid, and in both laboratories nicotinic acid uniformly failed to protect the animals. Day, Langston, and Darby (1938) and Day, Langston, Darby, Wahlin, and Mims (1940) reported data on fifteen monkeys which received from 4 to 40 mg. of nicotinic acid or amide daily. They all developed the typical syndrome of nutritional cytopenia: anemia, leucopenia, diarrhea, and eventual death. Topping and Fraser (1939) fed sixteen monkeys the Goldberger diet with ascorbic acid. Eight of them received 20 mg. weekly of nicotinic acid and eight received no nicotinic acid. There was no significant difference between the groups in the severity of the mouth lesions or in the survival period. (The average survival period of the eight monkeys receiving nicotinic acid was 77 days, of those not receiving nicotinic acid was 85 days.) It is impossible to explain the discrepancy between the results of Harris and the findings in the American laboratories. Day and coworkers and Topping and Fraser interpret their results as indicating that the monkey requires an additional unknown factor or factors (see section on Nutritional Cytopenia). It may be that some component of the diet used by Harris contained enough of this factor so that a nicotinic acid deficiency was the first deficiency to appear. For instance, it is not clear from the stated composition of his diet that the pea meal used was the cow-pea (California black-eyed pea) used by the Americans. The syndrome described by Harris probably does not have enough in common with human pellagra to fully justify the term "monkey pellagra," but his weight responses do offer evidence that nicotinic acid is required by the monkey.

Summary. Several experimental attempts to produce pellagra in the monkey are reported in the literature, but most of them antedate the discovery of the relation between nicotinic acid deficiency and human pellagra. Further studies are needed to clarify the rôle of nicotinic acid in the nutrition of primates other than man.

VII. NUTRITIONAL CYTOPENIA IN THE MONKEY—VITAMIN M

The rhesus monkey is peculiarly susceptible to a deficiency disease characterized by progressive anemia and leucopenia, lesions of the oral cavity, and diarrhea. Lowered resistance to infection, especially to bacillary dysentery, is a prominent feature. Other macaques (and possible other primates) are also susceptible to this deficiency syndrome, although most of the experimental work has been on the rhesus monkey (*Macaca mulatta*).

The deficiency manifestations cannot be prevented by the following substances, alone or in combination: protein of good quality, "complete" salt mixture, vitamin A, vitamin D, ascorbic acid, thiamin chloride, riboflavin, nicotinic acid, calcium pantothenate, pyridoxin, choline hydrochloride, inositol, or *p*-amino benzoic acid. It seems highly probable, therefore, that the deficiency of an unknown substance is involved. In the light of what we now know it is possible to read and interpret the literature, and we find that this syndrome, or certain manifestations of it at least, has been rediscovered and described every few years since 1919. The work has been approached from many angles, the diets used have been very different, the laboratories are widely scattered, but what appears to be the same syndrome has been found in each. The work has been reported from Conoor, Bombay, Little Rock, London, San Francisco, Chicago, Washington, Syracuse, and Columbus. In some cases the investigator was interested in (or recognized) only certain phases of the syndrome, and paid little or no attention to the other manifestations of the deficiency. Thus McCarrison's description (1919) was largely devoted to the intestinal signs—diarrhea, ulcerative colitis, and dysentery. He did not have facilities for making blood counts but he did report that his animals appeared very anemic. Wills and Bilimoria (1932), and Wills and Stewart (1935) were primarily interested in the anemia, although the leucopenia and diarrhea were mentioned. We (1935, 1938) described the leucopenia and anemia, the oral lesions, and the diarrhea, but at first we did not grasp the full significance of the dysenteric infection. We were, in fact, at one time on the point of abandoning the whole work because many of our experiments were complicated with bacillary dysentery. It only gradually dawned upon us that the dysenteric infection was not solely an accidental complication, but that the deficiency predisposed the monkeys to intestinal infections. Janota and Dack (1939) clearly demonstrated that the susceptibility to dysentery of monkeys receiving our diet 600 was definitely related to the deficiency syndrome.

Topping and Fraser (1939) and Tomlinson (1939) were primarily interested in the oral lesions, as were also Chapman and Harris (1941).

The Ohio group (Wilson, *et al.*, 1942; Saslaw, *et al.*, 1942, 1943), on the other hand, have placed most of their emphasis on the leucopenia and the susceptibility of the deficient monkeys to intestinal and respiratory infections.

The condition was referred to simply as "deficiency disease" by McCarrison (1919); as "macrocytic anemia" by Wills and Bilimoria (1932); as "leucopenia and anemia" by Day, *et al.* (1935); as "macrocytic nutritional anemia" by Wills and Stewart (1935); as "vitamin G deficiency" by Johnstone and Reed (1937); as "nutritional cytopenia" and "vitamin M deficiency" by Langston, *et al.* (1938) and Day, *et al.* (1938). Topping and Fraser (1939) referred to the syndrome simply as "B₂ complex" deficiency; Janota and Dack (1939) referred to the condition as "vitamin M" deficiency; and Chapman and Harris (1941) referred to it as "filtrate factor" deficiency. Saslaw, Schwab, Woolpert, and Wilson (1942) referred to the condition as "granulopenic leucopenia."

Regardless of the special interest of the investigators, however, it seems fairly clear that these groups of workers were all dealing with the same syndrome, which is characterized by leucopenia, anemia, oral lesions, diarrhea, ulcerations of the colon, and increased susceptibility to infections, particularly of the intestinal tract. The syndrome has not been entirely prevented by any combination of pure vitamins, and leads to a fatal termination unless the diet is corrected by the addition of crude liver extract, yeast, or other source of the nutritional factor.²

The various phases of the syndrome, the known distribution of the nutritional factor, and progress which has been made toward the elucidation of the problem, will be discussed in the following paragraphs.

Basal Diets Used. McCarrison (1919) used several deficient diets, designed to be deficient in vitamin A, vitamin B, or vitamin C, or combinations of two or more. At that time the existence of only those three vitamins was recognized. The basis of his diets was autoclaved rice, and no yeast was supplied in any of them, so the diets were probably all deficient in the factor essential to prevent nutritional cytopenia (except his normal control diet of natural foods). His monkeys developed diarrhea and dysentery on all the deficient diets.

The deficient diet of Wills and Bilimoria (1932) was designed after the diet in common use among Mohammedan women in Bombay. It consisted of polished rice, white bread, wheat chapatti, ghi (butter), white pumpkin, and meat. Certain of the animals received one or more of the following: orange, tomato or carrot, cod liver oil, milk, or yeast. The

² Since this manuscript was submitted to the editors, two papers by Waisman, *et al.* (1943, 1943a) have appeared which confirm in all important respects the findings of earlier investigations.

yeast was fed in doses of only one gram daily. Animals in all of the groups developed anemia; this was cured by liver extract or large doses of yeast (Marmite).

Day, Langston, and Shukers (1935) and Langston, Darby, Shukers, and Day (1938) used a diet of the following composition (diet 600): casein, vitamin-free, 10 g.; polished rice, 50 g.; whole wheat, 15 g.; salt mixture, 3 g.; sodium chloride, 2 g.; and cod liver oil, 3 g. Vitamin C was supplied by 4 g. orange daily in the early experiments and by 0.01 g. ascorbic acid daily in the later experiments. Suitable supplements of riboflavin and nicotinic acid were made to this diet. This diet, with certain pure vitamin supplements has been used by Janota and Dack (1939), and by the Ohio group (Wilson, *et al.*, 1942, 1943; Saslaw, *et al.*, 1942).

Day, Langston, Darby, Wahlin, and Mims (1940) used a modification of the Goldberger black tongue-producing diet, with ascorbic acid supplements. Whether supplemented with nicotinic acid and riboflavin or not, the typical syndrome of nutritional cytopenia developed. A modified Goldberger diet with ascorbic acid supplement was also used by Topping and Fraser (1939) and by Chapman and Harris (1941). Topping and Fraser used four groups of four animals each, feeding diets further supplemented as follows: (a) riboflavin and nicotinic acid, (b) nicotinic acid, (c) riboflavin, (d) no supplement. The response of the four groups was essentially the same; they all developed mouth lesions and diarrhea. "In many of these monkeys some anemia associated with a moderately severe leucopenia developed."

This response of monkeys to the feeding of the Goldberger diet brings up a very interesting point in comparative nutrition: dogs fed the Goldberger diet develop black-tongue; monkeys fed the Goldberger diet develop nutritional cytopenia. It would appear that man, in this particular respect, responds more like the dog than like the monkey. It remains to be seen, however, how common nutritional cytopenia is found in the human. Villada (1942) has reported numerous cases of what he considers to be clinical vitamin M deficiency, and their cure by feeding brewer's yeast or crude liver preparations.

The Blood Picture in Nutritional Cytopenia. The normal blood picture of the rhesus monkey has been studied extensively by numerous workers. Shukers, *et al.* (1938) reviewed the literature and tabulated a summary of the normal standards available up to that time. They added data on 150 complete hematological determinations on young normal rhesus monkeys. Ruch (1941) gives thirty-two citations to papers on hematology of monkeys and other primates. Recently Suarez, Rivera, and Morales (1942) have added valuable data on the normal blood count of various aged rhesus monkeys at the Santiago Primate Colony. The cell morpho-

logy of rhesus blood is very similar to that of man. The total erythrocyte count is somewhat higher, with an average slightly above 5 million cells per cu. mm. The normal total white cell counts are much higher than those of man, and are rather variable; the average for the young rhesus monkey is about 15,000 per cu. mm. with a normal range between 10,000 and 20,000.

In nutritional cytopenia in the monkey there is a progressive decrease in the numbers of all types of cells in peripheral blood. The picture varies from animal to animal; some show a pronounced anemia; others develop a profound leucopenia which is rapidly fatal, but show very little anemia. One of our monkeys (No. 47, Day, *et al.*, 1940) had a red cell count of less than one million per cu. mm., but that was an extreme case; minimum red counts between 2 and 3 million are more common. One monkey of Wills, Clutterbuck, and Evans (1937) showed an erythrocyte count of only 600,000 per cu. mm. Wills and Stewart (1935) referred to the syndrome as "macrocytic nutritional anemia," and they gave data showing that the volume index and mean cell diameter increased slightly. In the experience of this laboratory the hemoglobin and erythrocyte curves parallel each other with remarkable constancy (Day, *et al.*, 1940), and we consider it more proper to refer to the anemia in our animals as normocytic rather than macrocytic. The important point, however, is that it is not a hypochromic, microcytic type of anemia.

A profound leucopenia is the most characteristic feature of the syndrome, and the leucopenia involves all white blood cell types, although there is considerable variation from one to another animal with regard to the cell distribution. About the only generalization that can be made is to state that there is almost invariably an *absolute* neutropenia and an *absolute* lymphopenia; in some there is a *relative* increase in neutrophils and in others a relative increase in lymphocytes. A few animals have shown an almost complete agranulocytosis. Terminal total white cell counts are usually between 2,000 and 3,000 per cu. mm., which compared to an average normal of 15,000 is a profound leucopenia. Total white counts below 1,000 per cu. mm. have been found, but those cases are rare.

A few data on platelets indicate that a thrombocytopenia is also a feature of nutritional cytopenia in the monkey.

For details of the blood picture in the experimental condition, the reader is referred to papers by Wills and Bilimoria (1932), Wills and Stewart (1935), Day, *et al.*, (1935, 1938, 1940), Langston, *et al.* (1938), Wills, Clutterbuck, and Evans (1937), Wilson, *et al.* (1942) and Saslaw, *et al.* (1942). Other investigators have been more interested in other features of the syndrome (oral lesions, diarrhea, dysentery) and have only indicated that changes in the blood picture were found.

The addition of crude liver extract or yeast to the diet of an animal

with profound anemia and leucopenia is followed by a reticulocyte crisis, dramatic increase in white cells, and a slower return of red cell and hemoglobin levels to normal (see No. 47, Day, *et al.*, 1940; and Fig. 1 of Wills, Clutterbuck, and Evans, 1937). It is not possible to recover all animals, however; evidently certain animals reach what Wilson, *et al.* (1943) aptly term an "irreversible phase," at which time the addition of suitable supplements to the diet is ineffective. It is not known whether this "irreversible phase" is due to bone marrow exhaustion, or whether it is simply that the animal has been overwhelmed by an infection superimposed upon the deficiency.

Bone marrow studies by Wills and Bilimoria (1932) and Wills and Stewart (1935) indicate that the bone marrow in nutritional cytopenia is hyperplastic, the hyperplasia being of a megaloblastic type. On the other hand Wilson, *et al.* (1943) stated: "Bone marrow studies have shown a relative and absolute hypoplasia in the myeloid elements in the infected monkeys dying with peripheral leukopenia." Further studies of bone marrow in this condition should be made.

Oral Lesions in Nutritional Cytopenia. Mouth lesions, particularly of the margins of the gums, are among the most common signs of nutritional cytopenia in rhesus monkeys. Neither McCarrison (1919) nor Wills and Bilimoria (1932) mentioned such lesions; both of those laboratories used "*Macacus sinicus*" (the South Indian Bonnet Monkey) which may react somewhat differently in this regard from the rhesus monkey. McCarrison's monkeys were so wild that he did not handle them routinely, which is a more probable explanation of his failure to report mouth lesions. The first description of the mouth lesions was made by Day, Langston, and Shukers (1935): "The only consistent physical finding was an ulceration of the margins of the gums. This lesion usually developed within a short time after the onset of the leukopenia, first appearing as a slight recession of the gums, and later as a distinct yellowish ulceration. Upon wiping off the necrotic material with a cotton swab, mild bleeding resulted. This ulceration usually developed along the incisors first, and in several animals caused the teeth to fall out. In one monkey the ulcerated region was chiefly along the canine and molars, and progressed until a large area of bone was exposed. The gums were pale and there were no marked spontaneous hemorrhages. No other oral lesions were observed." Photographs of two monkeys with marked ulceration of the gums are given in Plate 50 in the report by Langston, *et al.* (1938). Few animals in this laboratory show such extensive gum changes, however.

Topping and Fraser (1939) and Tomlinson (1939) made a very careful study of mouth lesions in nutritional cytopenia and in experimental scurvy and vitamin A deficiency in the rhesus monkey. They used 16 monkeys

on diets deficient in the cytopenia-preventive factor. Although they placed their monkey in four groups, labelled "controls receiving flavin and nicotinic acid," "flavin-deficient," "nicotinic acid-deficient," and "flavin and nicotinic acid-deficient," it is important to point out that the monkeys in the four groups all developed the typical syndrome of nutritional cytopenia. The diets were all deficient in the cytopenia-preventive factor, and neither riboflavin nor nicotinic acid had any appreciable influence on the findings. With regard to mouth lesions their report is much more detailed and quantitative than any previous report. In addition to gingivitis and necrosis of the gums, they found extensive ulceration of the cheek in four of their 16 monkeys; in three of the four the ulceration of the cheek resulted in perforation and justified the term "noma." (In this laboratory we have had only one case of "noma" in approximately 80 animals developing nutritional cytopenia.) Smears from the gums of Topping and Fraser's monkeys showed an increase in fusiform and spiral organisms.

Chapman and Harris (1941) used diets almost identical with those of Topping and Fraser (1939), and found similar mouth lesions. In reading their paper, also, it is important to note that four of their diets were deficient in the cytopenia-preventive factor. Topping and Fraser and Chapman and Harris published excellent photographs of the mouth lesions.

Saslaw, *et al.* (1942) stated: "All monkeys on the 600 diet developed lesions of the oral mucous membranes which often became infected with streptococci, staphylococci or the flora of Vincent's angina. In monkeys on normal diets, no such lesions appeared spontaneously, and when accidental trauma of gingival or buccal mucosa occurred, no secondary infections were observed." The charts in the report by Day, *et al.* (1940) show that the gum necrosis appears *after* the leucopenia has existed for some time.

Edema in Nutritional Cytopenia. Edema has been reported as a frequent sign, but it is not as consistently seen as are the other signs of nutritional cytopenia. McCarrison (1919) saw no clinical evidence of "dropsy" in any of his monkeys. Since he had expected to produce beriberi on the diet he used, he no doubt looked for edema especially. Wills and Bilimoria (1932) did not mention edema. Both McCarrison's work and Wills and Bilimoria's work were on the Bonnet monkey ("*Macacus sinicus*"), while all later reports except one have been on work on rhesus monkeys.

Wills and Stewart (1935) stated: "In the later stages of the anaemia there was definite oedema, which varied in intensity from case to case." They then indicated that they believed it to be due to vitamin B₁ deficiency.

Day, Langston, and Shukers (1935), and Langston, *et al.* (1938) did not

mention edema, but Day, *et al.* (1940) reported that three out of the eighteen monkeys in that series exhibited marked edema. Since two of those three animals received 1 mg. of thiamin chloride daily throughout the experiment it appears impossible that the edema could be the result of vitamin B₁ deficiency. Edema was not mentioned by Janota and Dack (1939), Chapman and Harris (1941), Topping and Fraser (1939), Johnstone and Reed (1937), Wilson, *et al.* (1942, 1943) or Saslaw, *et al.* (1942). A few of Rao's monkeys (*Macacus sinicus*) developed edema of face, scrotum, hands, or feet (1942).

Since edema is not consistently found in nutritional cytopenia, its relation to the syndrome is still somewhat obscure. It seems certain, however, that the edema is not the result of thiamin deficiency.

Diarrhea, and Susceptibility of the Monkey to Bacillary Dysentery and Other Infections in Nutritional Cytopenia. Diarrhea develops in almost every monkey suffering from nutritional cytopenia. It is first evident as soft stools which later become watery, and are yellow or green in color. In some cases this watery diarrhea continues for weeks or months with little change. In others, blood and mucus appear, and the monkey becomes acutely ill with all the clinical manifestations of bacillary dysentery. Stool cultures taken at this stage usually reveal the presence of organisms of the genus *Shigella*. With the onset of dysentery the condition rapidly progresses to a fatal termination. This was first described by McCarrison (1919) who found amoebae in the stools of many of the monkeys, but who did not have facilities for making culture studies. Wills and Bilimoria (1932) reported the cure of diarrhea in one of their monkeys (No. 9) by treatment with liver extract. Day, Langston, and Shukers (1935) reported that with one exception their monkeys all developed a diarrhea within a short time after being placed on diet. Several of their animals developed clinical dysentery, and stool cultures revealed the presence of *Shigella paradysenteriae*, Hiss or Sonne variety. "Since the deficiency appeared to predispose to an attack of a latent dysentery, stool cultures were made at frequent intervals, and animals showing positive tests for pathogenic organisms were excluded from the series." Again in 1938 we called attention to diarrhea as a common finding in the syndrome (Langston, *et al.*, 1938).

It remained for Janota and Dack (1939), however, to point out the relation between "vitamin M" deficiency and susceptibility to bacillary dysentery in the monkey. Repeated failures to produce clinical dysentery by feeding large doses of living dysentery bacilli had led them to believe that some factor other than the bacterium was concerned in the production of dysentery. Verder and Petran (1937) had found that monkeys on a vitamin A-deficient diet developed bacillary dysentery. Janota and Dack

fed our vitamin M-deficient diet 600 (Langston, *et al.*, 1938) to a large group of monkeys. They found that control animals receiving an adequate diet did not develop dysentery, but that monkeys receiving the vitamin M-deficient diet spontaneously developed clinical dysentery and they were able to demonstrate the pathogenic bacilli in the stools of many of the animals. They stated: "These experiments would suggest that *Bact. dysenteriae* (Flexner) may live a saprophytic existence being present in the intestine in numbers which escape detection, even where a better type of selective medium . . . is used for isolation. The fact that the control monkeys did not develop dysentery nor did other experimental monkeys kept in the same room with the animals on a vitamin M-deficient diet indicates that the disease occurred as a result of lowered resistance due to vitamin deficiency and not due to contact infection."

Unpublished experiments in this laboratory have shown that the feeding of enormous numbers of living dysentery bacilli to a normal monkey receiving a normal diet (vitamin M-deficient diet supplemented with liver extract) did not produce diarrhea nor clinical dysentery, and had no influence on the blood picture. About a year later, however, this monkey was given a vitamin M-deficient diet, and he then developed nutritional cytopenia and succumbed to an acute attack of clinical dysentery. Editorially the Journal of the American Medical Association points out the possible relation of this to the development of human bacillary dysentery (Editorial, 1941).

Saslaw, *et al.* (1942) and Wilson, *et al.* (1943) have recently shown that vitamin M deficiency in the monkey results in "a markedly lowered clinical resistance to spontaneous infections with high mortality. The susceptibility to experimental infections with *Streptococcus hemolyticus*, Group C, and to influenza virus A, administered intranasally, was likewise increased in contrast with the controls on a normal diet."

Experiments upon the Properties and Chemical Nature of the Cytopenia-preventive Factor. It is now clear that the following substances will not prevent nutritional cytopenia in the monkey: vitamin A, vitamin D, ascorbic acid, thiamin chloride, riboflavin, nicotinic acid, pyridoxin hydrochloride, calcium pantothenate, *p*-aminobenzoic acid, choline chloride, inositol, pimelic acid, and glutamine (Day, *et al.*, 1940; Saslaw, *et al.*, 1942; Totter, *et al.*, 1943; Saslaw, *et al.*, 1943). No reports have appeared on the use of biotin in such experiments. The cytopenia-preventive factor (vitamin M) is evidently not identical with the pernicious anemia factor, since preparations highly potent in producing remissions in pernicious anemia are ineffective in curing or preventing nutritional cytopenia (Wills, Clutterbuck, and Evans, 1937, 1937a; Wills and Evans, 1938; Langston, *et al.*, 1938). The factor is not precipitated from solution by saturation

with ammonium sulfate; it is soluble in 70 per cent or 80 per cent alcohol but insoluble in absolute alcohol. It is not readily adsorbed on Fuller's earth (Wills, Clutterbuck, and Evans, 1937). It is destroyed by heating in air at 100°C. for 24 hours (Totter, *et al.*, 1943).

Wilson, *et al.* (1942) and Saslaw, *et al.* (1943) reported that a "folic acid" concentrate contains vitamin M. However, unpublished experiments in this laboratory (Totter and Mims) have shown that the basal vitamin M-deficient diets contain more "folic acid" (as measured by the growth stimulation of *Streptococcus lactis R*) than does the amount of yeast necessary to fully protect monkeys against nutritional cytopenia. Also, heating liver at 100°C. for 24 hours renders it ineffective in protecting the monkey against nutritional cytopenia, but does not appear to reduce its "folic acid" content. It therefore appears highly improbable that "folic acid" is identical with "vitamin M," if we define "folic acid" as the substance which stimulates *Streptococcus lactis R* under the conditions of the microbiological assay (Mitchell and Snell, 1941).

Recent experiments by Totter, *et al.* (1943) have shown that xanthopterin will produce a reticulocyte response and will increase white and red cell numbers in monkeys rendered cytopenic by a vitamin M-deficient diet. However, xanthopterin has not proved sufficient to maintain the white and red cell counts at normal levels indefinitely. They state: "The results suggest that xanthopterin is required by the monkey for hemocytopoiesis, but unidentified substances are also necessary to prevent vitamin M deficiency."

Distribution of Vitamin M. At the present time the only method for assay of the cytopenia-preventive factor is by experiments upon the monkey. It is possible that the leucopenia produced in rats by feeding them sulfanilylguanidine or succinyl sulfathiazole is the result of "vitamin M" deficiency, but if so it is yet to be proved. We shall probably have to await the chemical isolation and elucidation of vitamin M before small animal or microbiological methods can be used in its assay with any assurance of specificity. Using the monkey in assays of the distribution of vitamin M is made especially difficult since curative experiments are often unsatisfactory; the nutritional cytopenia results in such lowered resistance to infection that the recovery of animals is frequently impossible even with materials known to be potent. Furthermore, the time required to produce the deficiency syndrome is so variable that a preventive experiment is valid only if it covers an extended period. We consider that a supplement is fully protective only when it has prevented the appearance of the cytopenia for approximately a year.

Notwithstanding the obstacles in the way, some progress has been made in studying the distribution of vitamin M. Dried brewer's yeast is a good

source of the factor; 5 grams daily was fully protective but 2.5 grams daily was insufficient (Langston, *et al.*, 1938). On the basis of dry weight fresh liver is the most potent natural source of the factor—10 grams of fresh liver (beef) daily has proved fully adequate (unpublished experiments). Muscle meats (beef, pork) have not been fully protective even when fed at 20 grams per day level, although such quantities did appear to delay the onset of the cytopenia. Certain crude liver extracts have been shown to be excellent sources of the vitamin (Wills and Bilimoria, 1932; Langston, *et al.*, 1938; Day, *et al.*, 1940; Wills, *et al.*, 1937). Drying liver at 100°C. for 24 hours destroys its effectiveness, however (Totter, *et al.*, 1943). Crude acid-precipitated casein has been shown to be an unsatisfactory source of the factor when 10 grams daily were incorporated in the diet (unpublished experiments).

The grains and seeds which have been used in the deficient diets obviously are poor sources of vitamin M. Diet 600 (Langston, *et al.*, 1938) contains 50 g. polished rice and 15 g. whole wheat, per monkey per day. The Goldberger diet (Day, *et al.*, 1940; Topping and Fraser, 1939) supplies 40 g. whole corn and 5 g. cowpeas daily. It is therefore apparent that polished rice, whole wheat, whole white corn, and cowpeas are poor sources of the factor.

Orange is not a rich source of the vitamin, as evidenced by the fact that in our earlier experiments (Langston, *et al.*, 1938) we fed 4 grams daily as an antiscorbutic. The time of appearance of cytopenia and the survival period were not significantly changed when ascorbic acid was substituted for the orange. Twenty-five grams of banana daily has not been fully protective (Totter, *et al.*, 1943).

Summary. Young rhesus monkeys (and apparently also other macaques) when given a diet presumably adequate with regard to calories, minerals, and protein develop a syndrome characterized by anemia, leucopenia, oral lesions, diarrhea, and lowered resistance to infections, especially of the intestinal tract. The syndrome can be prevented by yeast or crude liver extract but the feeding of all the available pure vitamins has proved ineffective. There is some evidence that xanthopterin may be partially effective in preventing the deficiency disease. Some data are available on the distribution and properties of this factor, which has been termed "vitamin M." It is not known whether this factor is required by primates other than the genus *Macaca*.

VIII. PRIMATE CULTURE AND PRACTICAL DIETETICS

Published information on the care and feeding of primates is rather meagre. Much must have been learned about primate culture by scientific investigators and zoological garden curators which would be highly valuable

to others, but which has never been published. We have found Hartman's chapter "Housing and Care" (Hartman and Straus, 1933) very valuable. Ruch (1941) gives 24 references to papers on primate culture and methodology, a number of which give information on the feeding of primates. Unfortunately many of the papers referred to are not readily available. Katz and Katz (1936) report some very interesting, and amusing, experiments upon the feeding behavior and food selection of two species of monkeys. Carpenter's monograph on the behavior and social relations of the gibbon (1940) gives considerable information on the food of that species in its natural habitat.

IX. COMMON DISEASES OF CAPTIVE PRIMATES

Although this review deals with the nutrition of the primates, brief mention may well be made of some of the more common infectious and parasitic diseases which may be encountered in primates used as laboratory animals. The following references are not offered as a complete bibliography of primate diseases, but the papers referred to, together with the bibliographies contained in them, should be useful to an investigator initiating laboratory work on primates. Tuberculosis appears to be the most common disease of monkeys, and it is also found among the apes. Carpenter and Krakower (1941) stated that tuberculosis, dysentery, and fighting were the main causes of death in the monkeys of the Santiago Primate Colony during its first year. Kennard and Willner (1941) made the following statement: "In this colony (Laboratory of Physiology, Yale University School of Medicine), tuberculosis infects about one-fourth of all primates, and, once present, proceeds rapidly, within a period of months at the longest, to a fatal termination. There is no evidence of arrest of the disease at any time." Kennard and Willner (1941a) reported data on 216 autopsies on rhesus monkeys, in which they found the following: tuberculosis, 42; intestinal parasitism, 28; and pneumonia, 12 cases. The same authors reported findings in 70 autopsies on anthropoid apes. They gave the following causes of death of 58 chimpanzees: killed for experiment, 17; died as result of experimental procedure, 20; pneumonia, 9; diarrhea, 7; tuberculosis, 3; Vincent's infection, 1; and postpartum peritonitis, 1. Seven of them showed evidence of tuberculosis at autopsy, and intestinal parasites were found in three. Foster and Johnson (1939), Ronne and Sandground (1939), and Poindexter (1942) have reported studies of gastric and intestinal parasitism in captive monkeys.

X. SUMMARY

A search of the literature has revealed less than a hundred papers dealing with the nutrition of primates other than man: about half of those have

appeared within the past eight years. Most of the experimental studies on the nutritional requirements of primates have been upon the rhesus monkey. Several investigators have restricted monkeys to a diet deficient in vitamin A, but in only a few of the animals were the classic eye signs of vitamin A deficiency observed. Spontaneous rickets has been observed in young monkeys and the disease has also been experimentally produced by feeding a diet deficient in vitamin D; the condition closely resembles rickets in the human infant. Monkeys also appear to be susceptible to osteomalacia. Experimental calcium deficiency has been produced in rhesus monkeys by the prolonged feeding of a diet deficient in this element, and a syndrome developed which was characterized by irritability, anorexia, weakness and muscular atrophy, and reduction in the calcium content of the bones. Neither the vitamin A or vitamin D requirement of the monkey (or other primate species) has been determined.

Young rhesus monkeys are readily susceptible to scurvy, and the disease is identical with human infantile scurvy. The minimum daily ascorbic acid requirement of a young rhesus monkey appears to be 2 mg. or less; the requirement for optimum nutrition has not been established. No record has been found of experiments upon ascorbic acid deficiency in other non-human primates.

Spontaneous beriberi has been observed in monkeys, but attempts to produce it experimentally by dietary means have not been uniformly successful. There is no information available on the thiamin requirement of monkeys and other primate species. The data on nicotinic acid deficiency in the monkey are too few to be conclusive.

Rhesus monkeys (and other macaques) when given a diet deficient in the B vitamins develop a syndrome characterized by anemia, leucopenia, oral lesions, diarrhea, and susceptibility to intestinal infections. The syndrome cannot be prevented or cured by any combination of the now available pure vitamins, but it can be prevented, and sometimes cured, by yeast and certain crude liver extracts. This nutritional factor awaits chemical identification.

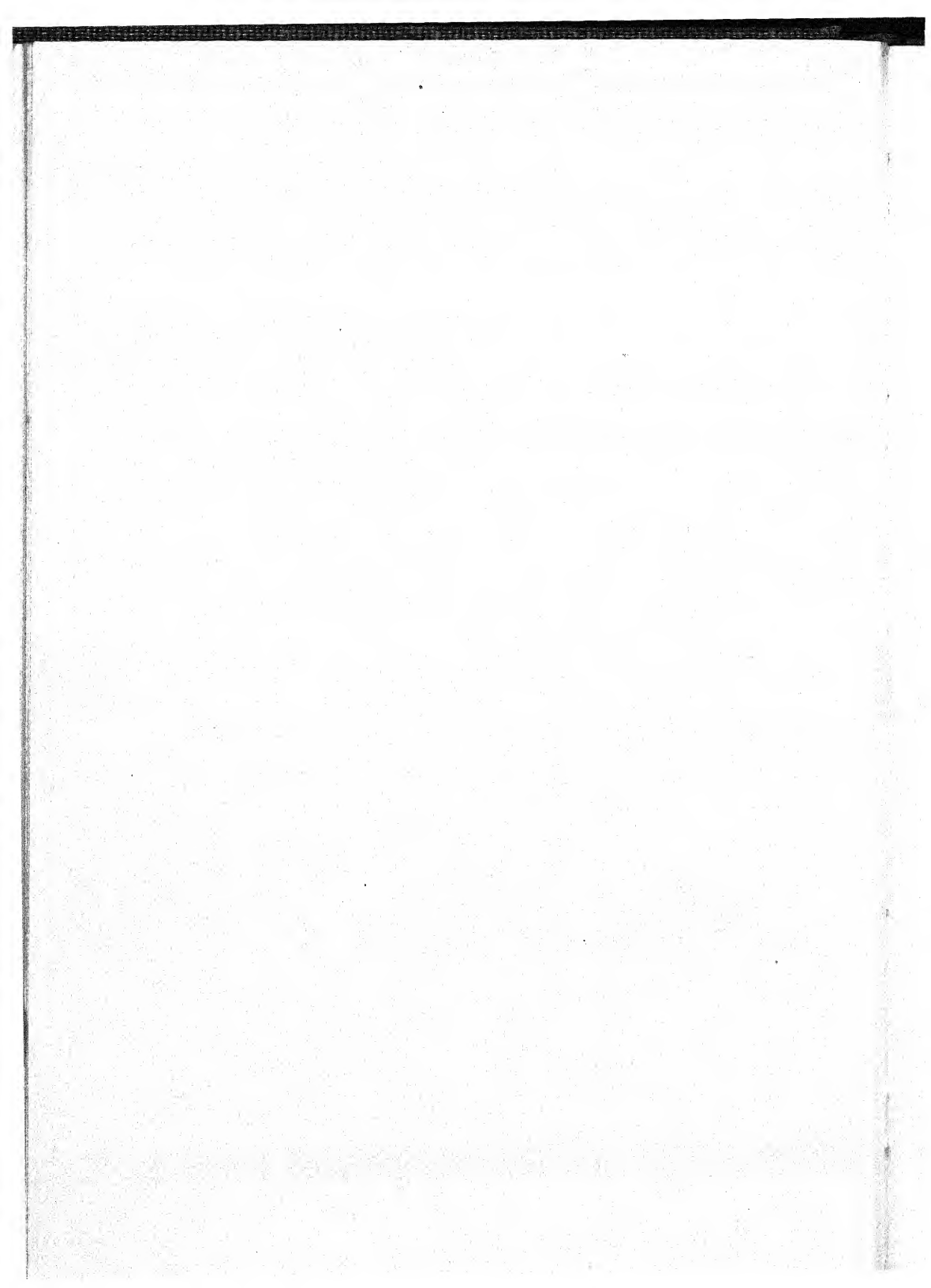
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Physiological Action of Vitamin E and Its Homologues

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I. INTRODUCTION

Despite more than two decades of research vitamin E still remains the mysterious intruder in the vitamin family, accepted at first with much reluctance and later reared somewhat as an outcast largely because of failure to satisfactorily establish its practical importance for man and beast. Its chemical nature is known, its laboratory synthesis accomplished, its wide distribution in the plant and animal world recognized, and

the effects of its absence extensively studied in a large series of laboratory animals. Its physiological rôle in the animal body is still a matter of conjecture.

The scientific literature on the subject of vitamin E is vast, especially that of the past twelve years. Much of it is contradictory, some of it merely baffling. There have already been presented a series of excellent reviews of progress in this field; some of them general in nature (12, 82, 116, 125, 159, 221), others dealing more specifically with the chemical nature of the vitamin (119, 120, 204) and the tissue changes caused by its absence (83, 154, 181, 184, 226). The purpose of the present review is two-fold: first, to cull from this vast body of knowledge those observations and deductions which, in the opinion of the reviewer, may best serve as a firm foundation from which may eventually arise a true understanding of the physiological rôle of vitamin E in the animal organism; second, to attempt to indicate those trends of investigation, either in progress or suggested by observations at hand, which offer the most promising approach to this desired goal.

Preliminary to this, it may be helpful to outline briefly the changing trends of interest and progressive developments as the story of vitamin E has been unfolded since 1923, when convincing evidence concerning the existence of this vitamin was first presented. During the following eight years attention was devoted chiefly to a study of (1) the effects induced in the female and male reproductive systems of the rat; (2) its distribution in a wide variety of plant and animal tissues by application of bio-assay tests; (3) its relation to late-lactation paralysis in young rats and to growth retardation in adult rats; and (4) its chemical concentration from wheat germ oil.

With the indication, in 1931, that a state of nutritional muscular dystrophy occurs in guinea pigs and rabbits deprived of vitamin E (96), the response of a wide variety of animal types was investigated. Incidental to these studies arose perplexing interrelationships between vitamin E and dietary fats which are still receiving much attention. Parallel and rapid developments along chemical lines led in 1936 to the chemical isolation of α -tocopherol as the prototype of vitamin E (87), and to the elucidation of its chemical structure (90) and its chemical synthesis (122, 208) within the next two years. Since then, abundance of the pure vitamin has greatly facilitated studies of all types, especially those concerned with the specificity of the anatomical and biochemical lesions of E deficiency.

Interest which simultaneously developed in the relation of vitamin E to lesions of the nervous system, to dysfunction of certain endocrine organs, and to habitual abortion and related disorders in man and domestic animals has declined, largely due to lack of support from subsequent studies. On

the other hand, the nutritional muscular dystrophies have received wide attention, paralleled by an intense but fruitless search for evidence of a homologous clinical entity in man and, more recently, by studies indicating a relationship of vitamin E to structural or functional states of the vascular system in certain species. Considerable progress has also been made in correlating chemical structure with biological action, and in analyzing the metabolic dysfunctions of the deficiency state and their response to tocopherol.

II. THE NATURE OF VITAMIN E

1. *Chemistry of the Tocopherols*

The ability of plants to prevent the symptoms of vitamin E deficiency in animals is due solely to the presence of three higher alcohols for which the common designation of tocopherol (tokos-offspring; pherein-to bear; -ol indicating the alcoholic nature) has been given (87). None of the other organic compounds known to possess vitamin E action is found in the plant world. The methods used for their isolation from natural sources involved extraction with fat solvents, saponification, partition of the non-saponifiable fraction between solvents, removal of sterols with digitonin, chromatographic analysis, vacuum distillation, and isolation as crystalline allophanates or as other esters. Molecular distillation from vegetable oils is used extensively for the commercial production of the natural tocopherols; the synthetic forms are readily prepared in the commercial laboratory. The individual tocopherols differ in their solubilities and can be separated from each other by fractional crystallization of their esters, or by differential absorption. The free tocopherols are viscous oils, which probably represents the state in which they occur in plant sources.

Two highly purified preparations of natural tocopherols (25) have recently been obtained in crystalline form (191a) by Dr. Charles D. Robeson, of Distillation Products Inc., Rochester, N. Y., to whom the writer is greatly indebted for the first recorded photographs of crystalline alpha tocopherol (Fig. 1) and gamma tocopherol (Fig. 2). Both compounds were crystallized from a 2.5% methyl alcohol solution at -35°C . and were dried at -10°C . under high vacuum. Attempts to crystallize natural beta tocopherol were unsuccessful.

Beta (II) and gamma (III) tocopherols have the empirical formula $\text{C}_{23}\text{H}_{43}\text{O}_2$, being isomers which differ only in the position of one CH_3 radical, and are lower homologues of alpha tocopherol (I); the latter having an additional CH_3 group in the benzene ring and the empirical formula $\text{C}_{29}\text{H}_{50}\text{O}_2$. Their laboratory synthesis, which may follow essentially the same pattern as that in plants, involves the interaction, with or without a

catalyst, between phytol compounds (phytol, phytyl halides, or phytadiene) and hydroquinones, a reaction which apparently cannot be carried out



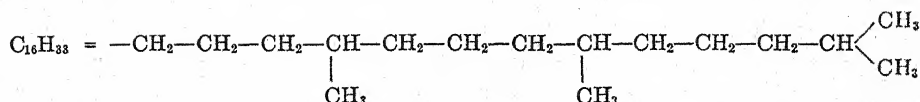
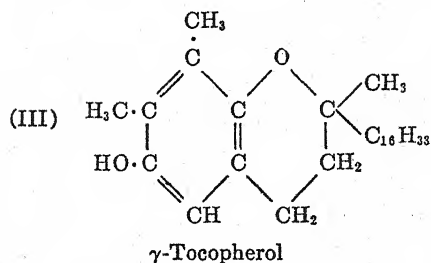
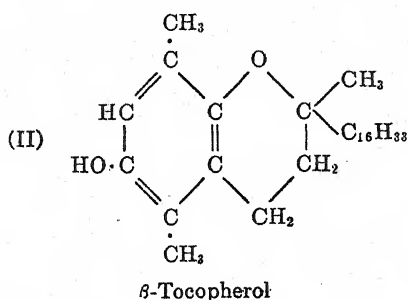
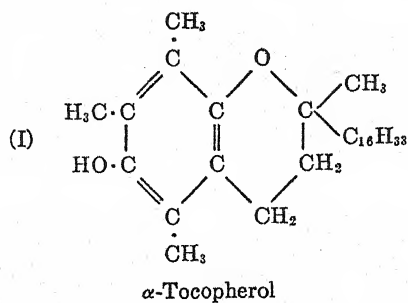
FIG. 1—Natural α Tocopherol $\times 30$



FIG. 2—Natural γ Tocopherol $\times 10.5$

(Photomicrographs, Figs. 1 and 2, courtesy of Mr. R. P. Loveland, Eastman Kodak Company, Research Laboratories).

in the animal body. Only recently has synthesis been accomplished by the use of compounds in which phytol is not an intermediate (117, 207).



The tocopherols have a characteristic absorption spectrum in the ultra-violet and a characteristic extinction coefficient. They are sensitive to ultraviolet light, fairly stable to visible light, soluble in lipid solvents, practically insoluble in H_2O , stable to 200°C . in the absence of O_2 and to 100°C . in H_2SO_4 and HCl , destroyed slowly by alkalis and highly sensitive to oxidation. Their esters, especially their succinates and acetates, are much more stable than the free forms. Neither the free tocopherols nor their esters are toxic to animals (61. 25).

These tocopherols are not merely by-products of plant metabolism but represent members of a large group of "inhibitols" which are responsible for the stability of vegetable fats toward oxidative deterioration (176). They are remarkably effective as stabilizers of animal fats and unsaturated fatty acids *in vitro*, which is probably not their primary rôle in animal economy, and are themselves very susceptible to oxidation unless protected by other antioxidants. Their antioxidant activity, which is also possessed by their biologically inactive allophanates but not by their simple esters, is attributed to the presence of a free phenolic hydroxyl group. The biological activity of the three tocopherols, as measured by the prevention of fetal resorption in rats, is inversely proportional to their antioxidant activity (175) and is dependent upon other characteristics of the molecule (p. 120).

Although α -tocopherol is the prototype of vitamin E, in the discussion to follow the term vitamin E will be used rather generally to indicate any or all of the tocopherols unless otherwise specified.

2. Chemical and Physical Tests

The methods devised for the chemical or physical detection and measurement of tocopherols differ primarily in the procedure used for estimating their oxidation products (quinones) after treatment with various oxidizing reagents. They are briefly as follows: spectroscopic analysis after oxidation with alcoholic silver nitrate (49); potentiometric estimation after gold chloride treatment (121); colorimetric measurement after oxidation with alcoholic nitric acid (92); colorimetric measurement after oxidation with ferric chloride and conversion of the ferrous ions into a red complex by the addition of α , α' -dipyridyl (77); colorimetric oxidation-reduction method permitting differentiation between tocopherols and their oxidation quinones, based on methods used for vitamin K (195); and amperometric titration at the dropping mercury electrode after gold chloride oxidation (205).

The methods possess certain disadvantages when applied to natural sources of the vitamin. Most require a rather concentrated source of the vitamin, often necessitating preliminary saponification which may cause loss unless proper precautions are taken. Some are affected by the presence of other oxidation products and carotenoids. None can reveal the relative concentration of α -, β -, and γ -tocopherols which differ in their biological activity; hence, all should overestimate the biological activities of tocopherol mixtures. This is not always the case when dealing with wheat germ oil (203) or animal tissues (64). Of the various methods listed that of Emmerie and Engel (77) has been most extensively used, after modifications designed to permit its better application to plant oils (56, 187), blood (220, 224), and animal tissues (64, 107a). The last two methods

mentioned above (195, 205), which are of recent origin, possess certain advantages over the others listed but have not been extensively tested on a variety of materials. Although chemical (64) and biological (153) determinations of E in rat muscle have shown fair agreement, much more information is necessary to establish the extent to which the chemical and physical methods can replace the laborious and expensive biological assay procedure, especially when dealing with animal tissues and fluids. Much depends upon the ability of future investigations to establish whether the biologically less active β - and γ -tocopherols actually exist as such in animal tissues.

3. Biological Assay

The biological method for assay of vitamin E, based upon the ability of the vitamin to prevent fetal resorption in female rats as developed by Evans and Burr (84), has undergone considerable modification during the past five years. The frequent failure of vitamin E deficient rats to resorb during their first pregnancy ("first litter fertility"), which for many years necessitated the establishment of a proven resorption in each rat used for assay tests, was due primarily to vitamin storage in young rats having access to a stock diet moderately rich in E prior to weaning; traces of E in the purified diets and placental and mammary transfer of the vitamin having little to do with this troublesome phenomenon (157, 158). The extensive use of low-E stock diets such as the commercial dog chows, and the increasing tendency to transfer breeding females and offspring to the deficient diet during the last week of lactation, have permitted the utilization of virgin rats for bio-assay tests. On the other hand, there exists a deplorable lack of uniformity in those phases of the bio-assay test concerned with the time and duration of feeding of the test dose and the criteria of positive response.

Single doses of vitamin E are increasingly effective as the time of administration approaches the 10th day of pregnancy, and are less effective when fed on the 4th or 5th day than when the same total dose is distributed over the first ten days of pregnancy (149). Obviously a single dose procedure is the simplest and most satisfactory when dealing with a concentrated preparation of vitamin E. On the other hand, when assaying animal or plant tissues the total amount of material necessary to provide a curative dose may be of such magnitude as to require 10 days of daily feeding. The experimental establishment of a scale of ratios between the total dose fed and the duration of feeding might have real merit.

The International Rat Unit of vitamin E, provisionally represented by 1.0 mg. of synthetic *d,l*- α -tocopherol acetate, implies that level of biological activity of any substance which when administered orally will prevent

resorption in 50% of a reasonable number of female E-deficient rats (113, 114). Although the biological response is a graded response, and hopes were once held that it might be evaluated as such (149), it seems necessary to treat it as an "all or none" type (152). A critical analysis of various aspects of the bio-assay question has been presented by Bacharach, *et al.* (13, 14, 17).

As the assay test is applied in different laboratories, considerable differences exist in regard to the criteria of positive response. The following criteria have been proposed: the delivery of one or more living offspring at term (84); the percentage of placental implants resulting in birth of living offspring (178); the delivery of one or more offspring at term, either living or dead (17); the presence of two or more viable fetuses on the 16th day of gestation (115, 152); increases in maternal body weight during gestation (102).

The use of the same rat for successive assay tests does not give trustworthy results. Ineffective doses previously administered may favorably sensitize the animal for a subsequent test. Furthermore, the vitamin E requirements for successful pregnancy increase with successive gestations (75). Particularly important is the necessity for gross inspection of the uterus during or immediately after an assay pregnancy in order that instances of pseudopregnancy with uterine bleeding (which occur frequently but irregularly and can easily be confused with resorption pregnancies) are not misinterpreted as negative responses to dose (152). This phenomenon has been overlooked by most investigators.

Lack of uniformity in time and duration of dosage, in the criteria of response used, and perhaps in the quality and quantity of fats in the experimental diets, undoubtedly accounts for the discrepancies in the results reported (113, 114) from cooperative assays on the provisional International Standard for vitamin E (synthetic racemic *d,l*- α -tocopherol acetate). Results from nine different laboratories indicated a median fertility dose varying from 0.56 mg. to 1.71 mg. It is to be hoped that a uniform bio-assay procedure will be followed, and consideration be given to the adoption of a stable crystalline ester of α -tocopherol as the Reference Standard, when the question of the International Unit for vitamin E is re-examined.

The possible use of exudative diathesis symptoms in chicks (56) and the creatinuria of nutritional muscular dystrophy in rabbits (140) as a measure of biological response has not been extensively explored. Careful comparisons between results obtained by certain of these methods and those by the rat antisterility test might prove quite informative, as indicated by the limited data now available (see Table IV, p. 143).

4. Distribution in Plants

Due to the laboriousness of the bio-assay method, and the limitations of chemical methods when applied to plant and animal tissues, there exists no exhaustive analysis of the relative concentration of vitamin E in food-stuffs such as have been presented for other vitamins. However, the difficulties encountered in producing the deficiency state with diets containing small amounts of natural foods and information obtained by assay tests presuppose a wide distribution of the vitamin in plant and animal tissues. All green plants so far examined by direct methods have contained demonstrable amounts. Although the germ of cereal grains is considered the richest source of E there is some evidence (56) that green leafy vegetables and rose hips, on the basis of dry weight or fat-extractable material, may contain even more tocopherol than wheat germ.

The three tocopherols, which are the only known substances in the plant world possessing vitamin E activity, occur in the free form rather than as esters (172), at least in the seed oils, and vary widely in the relative proportion in which they exist in different plants. For example, California wheat germ oil contains about twice as much α - as β -tocopherol (70), together with traces of the γ -form, whereas slightly less β - than α -(172) as well as a great preponderance of β -tocopherol (214) has been reported in European oils. Cottonseed oil sometimes contains as much γ - as α -, but usually much less (70), while the tocopherol of corn oil is almost exclusively of the γ -type (71). Lettuce contains only α -tocopherol. No satisfactory explanation has been offered for this variable distribution of the three tocopherols in plants. Their storage in the embryo of the seed suggests that they represent stabilizers of fats during the period of dormancy or a reserve storage for some special function in early development and growth.

The existence of vitamin E in lower plants such as the algae, fungi, liverworts, mosses, ferns, and primitive seed plants has not been demonstrated. The assumption that it does not exist in plants lacking chlorophyll receives support from its reported absence in the fungus *Phycomyces* (194). While it is true that information regarding the distribution of carotene and of vitamin K in plants has not greatly enriched our knowledge of their physiological rôle in either plants or animals, it does not necessarily follow that extension of our knowledge of the distribution of tocopherols in the plant world would not offer additional clues as to their physiological action in living cells.

5. Distribution in Animals

Except for the suggested need of vitamin E for growth of ovaries and embryos in the crustacean *Daphnia magna*, on the basis of which a biologi-

cal assay test was once proposed (219), no evidence exists that the tocopherols are required by, or stored by, any invertebrate animals. Although vitamin E has been demonstrated in tuna fish oil (32), and α -tocopherol isolated from shark liver oil (192), there is general agreement that commercial cod liver oil is devoid of E activity. Other evidence that the tocopherols are present, and may play an important rôle, in the tissues of cold blooded vertebrates lies in the reports that integrity of the testis and striated musculature of the guppy fish (45, 46), and the thyrotropic and other functions of the pituitary gland in frog tadpoles (188) are dependent upon a dietary source of vitamin E. The avian species (chick, duck, and turkey) exhibit a pronounced need for vitamin E, especially during early embryonic growth (1) and early maturity (186). Egg yolk contains considerable vitamin E, the amount varying with the dietary intake of the pullet (20).

Only in mammals has some attention been given to the distribution of the vitamin in different tissues, in the hope of gaining some insight into the manner in which the mammalian organism utilizes and stores the vitamin. Bio-assays of fresh tissues from rats receiving approximately 4 and 100 times the minimal daily requirement of vitamin E (153) indicate the following: at the lower level of intake the liver stored about one-half to one-fourth as much vitamin E (per gram of fresh tissue) as the skeletal muscles, body fat, and visceral organs; at the higher level of intake the liver stored 14 times, the other tissues only 3 to 4.5 times, as much as at the lower intake; total storage in rats at the low and high levels of intake, respectively, was estimated to be about 3 and 14 median fertility doses; limited assays on rats receiving 10,000 times the minimal intake revealed a storage in liver 150 times, and in skeletal muscle 12 times, that found at the low level of intake (Table I).

It appears that the liver, whose storage of vitamin E is sacrificed to enable other body tissues to meet their needs when the intake is low, constitutes the chief repository for the vitamin when the intake is considerably increased. Although the rat liver is strikingly limited in its ability to store vitamin E when compared to the storage of other fat-soluble vitamins, it constitutes the best index of previous intake. Appreciable quantities of vitamin E have also been demonstrated by bio-assay in human and monkey livers but not in dogs with bile fistulae (153), the latter finding confirming the observation that rats (103) and dogs (36) with bile fistulae cannot absorb vitamin E from dietary sources.

Regardless of how high the maternal intake of E may be, and the fact that the uterus and placenta contain as much vitamin as most other body tissues, the newborn rat begins life with a negligible supply of the vitamin. However, after 24-48 hours of lactation the concentration of E

per gram of tissue (including undigested curd in the stomach) increases about four-fold and approximates that in the body tissues of the mother (153). Whether the colostrum milk is richer in the vitamin than later milk has not been determined.

It is apparent that substances possessing biological activity of vitamin E are widely distributed throughout the tissues of the rat and show no obvious predilection for those tissues which suffer pathological alteration after E deprivation. Furthermore, the total storage in the animal body represents but a small fraction of the total intake, which is in accord with

TABLE I
Estimated α -Tocopherol in Fresh Tissues of Low-E and High-E rats

Tissue	mg. α -tocopherol per kg. tissue (based on 1 mg. α -tocopherol = M.F.D.)		
	Low E (4 \times minimal)	High E (100 \times minimal)	Excess E (10,000 \times minimal)
Liver.....	8.3	118	1000
Muscle.....	16.6	67	200
Kidney.....	16.6	77	
Body fat.....	20	59	125*
Pancreas and thymus.....	22	100*	
Heart.....	28.6	111	
Lung.....	28.6	125	
Spleen.....	33.3	100*	
Testis.....	16.6	50*	
Epididymis.....	18*	67*	
Prost. and Sem. Ves.....	16.6*	50*	
Uterus.....		125	
Placenta.....		77	
Mammary gland.....	28.6*	250	
Newborn.....	5	20	
Suckling young (24-48 hrs.).....	20	67	

* Values based on limited number of assay tests.

earlier analyses of the body fat, muscles, urine, feces, and blood of rats by the spectroscopic methods leading to the conclusion that the tocopherol molecule is readily broken down or ineffectively stored by the animal organism (49, 166).

Chemical tests have demonstrated tocopherols in dog and human blood serum before and after tocopherol feeding (220, 224) but have received only limited application clinically. That about one half the tocopherol in serum may exist in quinone or oxidized form (195) is of particular interest. Chemical assays of liver, muscle, and kidney of domestic animals (123) have given rather low tocopherol values compared with those ob-

tained by Devlin and Mattill (64) on normal rat muscle. The latter compare favorably with biological determinations (Table I), especially if it could be assumed that the tocopherol of rat muscle exists entirely or predominantly in the α -form rather than as a mixture. However, it is not known whether all three tocopherols retain their individual identity in the animal body as they do in plants. The lower values by chemical tests and particularly Devlin and Mattill's failure to find very significant reductions in muscle tocopherol in deficient rats except after prolonged E depletion, suggest that at least a portion of the tocopherols may be combined in the cells in such a manner as to resist the oxidizing reagents. There is also evidence that some of the tocopherol present in muscle resists extraction by simple organic solvents (107a) such as used in the chemical assays.

Recent modifications of the chemical test as applied to animal tissues have enabled Hines and Mattill (107a) to confirm the greater storage of the vitamin in the liver than in the musculature (of rats and rabbits), and also its absence in the urine but its abundance in the feces of rats, given a high tocopherol intake. Their failure to find evidence of tocopherylquinone in either liver, muscle, or urine is also worthy of note.

6. Biological Activity

(a) *The Tocopherols and Their Esters.* Assays reported from different laboratories have indicated that the antisterility potency of α -tocopherol is about twice that of β -tocopherol, and twice to four times that of γ -tocopherol. The purity of samples of the latter is questionable, because of the difficulty in separating it from α -tocopherol. Since many factors in the assay procedures make close comparisons impossible, no effort will be made to review the isolated reports on this question. The data presented in Table II, based upon a table recently compiled by Hickman (106), represent the best summary of our present knowledge. Although the relative median fertility dose (M.F.D.) of natural and synthetic tocopherol is taken as unity, the actual m.f.d. is approximately 0.75 mg. The assays of Joffe and Harris, on which much of the information in Table II is based, have been reported in part elsewhere (115). Many of the same compounds were simultaneously assayed by the writer with essentially the same result; there being consistently a slightly greater effectiveness which seems related more to distribution of the total dose over the first 10 days of pregnancy (as compared to the 4th to 6th days in the Joffe and Harris tests) rather than to response differences in the experimental animals. The writer's assays of synthetic β -tocopherol and synthetic γ -tocopherol (Merck) indicated considerably greater biological activity than recorded for these compounds in Table II, the reasons for which are not yet clear. Some

investigators have reported that natural γ -tocopherol has about one third (73) or one-fourth (154) the potency of natural α -tocopherol; others that it is as active as β -tocopherol (192). These findings now appear to be attributable to the presence of α -tocopherol as a contaminant (115). These assays, and practically all those reported in the literature, involve the use of diets containing moderate amounts of added fats; a fact to be noted in view of recently reported evidence that biological activity of tocopherols is increased when diets containing no added fats are used in assay tests (102).

TABLE II
Biological Activity of Tocopherols and Esters

Natural tocopherols and their esters			Synthetic tocopherols and their esters		
Tocopherol	Physical characteristics	Relative M.F.D., on basis of tocopherol content	Tocopherol	Physical characteristics	Relative M.F.D., on basis of tocopherol content
α -	Viscous oil	1	α -	Viscous oil	1
β -	" "	2.5	β -	" "	5
γ -	" "	12	γ -	" "	20
α -acetate	Needles	1	α -acetate	" "	1
α -palmitate	Glassy laths	12	α -palmitate	White granules	
α -acid succinate	Needles	1	α -calcium succinate	White solid	1
β -azobenzene carboxylate	Orange rosettes	8	α -phosphate (Na salt)	" "	1
γ -palmitate	Glassy laths	12	α -tocopherylamine (hydrochloride)	" "	1
α -allophanate	" "	0			
β -allophanate	Colorless laths	0			
γ -allophanate	White granules	0			

Some reports in the literature have indicated that certain esters of the tocopherols are biologically more active than the corresponding free forms. This difference now appears to be due to the greater stability of the former; the conclusion being that the free tocopherols are in no case less active, though they are frequently more active, than the tocopherol equivalent of their esters. The allophanate esters show no biological activity. Certain esters, such as the α -acid succinate ester, are crystalline and relatively easy to purify and handle, by virtue of which they offer a more suitable International Standard than the oily compound, *d,l*- α -tocopherol acetate provisionally accepted (106, 115).

It has been generally accepted that natural (*d*) α -tocopherol possesses

the same biological activity in the rat assay test as does synthetic *d,l*- α -tocopherol (78). However, recent evidence of a somewhat indirect nature (103a) indicates that highly purified samples of natural α - and β -tocopherols are approximately twice as active as their synthetic *d,l* forms. It is to be hoped that the optical isomers of the synthetic tocopherols will soon be isolated and the relative biological activity of the *d*- and *l*-forms tested directly.

Although there is need for more information concerning the relative effectiveness of α -, β - and γ -tocopherols in preventing resorption in female rats, much more attention should be given to the activity of these compounds in preventing other manifestations of vitamin E deficiency in the rat and in other laboratory animals. In most instances we have merely the observation that the lesions are preventable by natural or synthetic α -tocopherol, in free or ester form, supplemented by a few efforts to establish the minimal daily requirements for preventing nutritional muscular dystrophy in the rat (97) and rabbit (80). Exclusive of the rat sterility test, β -tocopherol (synthetic) has been tested only in the chick, one study (56) reporting it quite ineffective in protecting against exudative diathesis at levels of dosage sixteen times the effective dose of synthetic α -tocopherol, though shown to be one sixth as active as the latter when tested on rats; another study (29) finding it to be about one half as active as α -tocopherol in preventing edema in chicks. Further studies along such lines should yield highly interesting and significant results.

b) *Other Compounds*. The lack of chemical specificity of vitamin E is remarkable. Relatively slight modification in the structure of α -tocopherol may abolish the biological activity whereas compounds only slightly related to the tocopherols may be active. Approximately 150 compounds have been prepared and tested of which about one-third have shown variable degrees of antisterility activity. They represent widely different groups of organic compounds—chromans, coumarans, coumarins, phenols, quinones, hydroquinones, and their esters and ethers. None is more potent than α -tocopherol, and many are effective only when fed at levels of 50–100 mg., which often approaches the toxic level. The assay data up to 1939 have been summarized by Evans, *et al.* (88), and an excellent review of the literature has been presented by Smith (204). Various compounds prepared and tested since these reviews appeared have added little to the question of chemical structure versus function.

Biological activity of α -tocopherol is reduced by shortening of the long aliphatic side chain or replacing it with other groups, by changing the number or position of the methyl groups in the benzene ring (a situation analogous to that of riboflavin), by replacement of the methyl by ethyl groups, and by removal of the phenolic hydroxyl group or by masking it

as an ether or allophanate (88, 120, 204). Masking this hydroxyl group as a carboxylic ester or as an amine has little or no effect on potency (206), suggesting that biological activity is related in part to the ability of the animal body to bring about chemical reactions at the position of this hydroxyl group. Contrary to earlier reports (72, 88), α -tocopherylquinone, produced from α -tocopherol by mild oxidation, is biologically inactive in rats (99, 190, 229) and guinea pigs (100). Although this mitigates against the interesting suggestion that tocopherols exert their biological activity by participating directly in a reversible oxidation-reduction system in the body, the quinone cannot be entirely eliminated as a possible agent in biological actions (206).

III. STRUCTURAL AND FUNCTIONAL DISTURBANCES DUE TO LACK OF VITAMIN E

1. *Differences in Species*

The most outstanding characteristic of experimental vitamin E deficiency, which has no parallel in any other vitamin deficiency state, is the multiplicity of structural and functional alterations exhibited sometimes by closely related species, and the similarity of certain changes in quite unrelated species. In some deficiency states an understanding of the morphological changes occurring in cells and tissues as a direct result of the particular vitamin deficiency, and during the recovery period following therapy, constitutes a valuable guide to the physiologist and biochemist in their approach to the problem of vitamin functions. This is particularly true of deficiency of vitamins A, D, and C, as emphasized by Wolbach and Bessey (226) in a recent and comprehensive review of the tissue changes in different vitamin deficiencies. In the case of vitamin E deficiency the situation is more complex. The pattern of change is not limited to a specific type of cell or tissue, and therapy is not followed by an orderly process of structural repair. Morphological changes occur in a variety of unrelated tissues, seem not to be related to dysfunction of any specific cell type, and differ from those of other deficiency states in their apparent irreversibility; such that repair can be spoken of only in physiological or biochemical terms to indicate the ability of affected tissues to resume function despite irreparable loss of certain structural units.

Those structural and functional derangements which seem to be most characteristic of the deficiency state are tabulated in Table III in such a manner as to emphasize the type of body tissue disturbance which, in the light of present knowledge, seems to best explain the manifestations of vitamin E deficiency. In some instances the latter are predominantly biochemical and represented by prominent symptoms which cannot be

directly related to structural changes in a particular type of cell or tissue, as in the case of nutritional encephalomalacia and exudative diathesis in the chick, which, though listed under the vascular system on the basis of associated capillary dysfunction, may constitute merely the reflection of

TABLE III
Tissue Dysfunctions in Vitamin E Deficiency

Animal Type	Dystrophy of Skeletal Muscle	Changes in Smooth Muscle	Vascular System	Reproductive System and Embryo
Rat				
-fetus			Vascular stasis and hemorrhage	Resorption
-nursling	acute			
-adult	chronic	Necrosis of uterus and sem. ves.		Testis degen.
Mouse				
-fetus				Resorption
-nursling	slight			
-adult	slight			
Guinea pig	acute			Testis degen.
Rabbit	acute			
Hamster	acute			Testis degen.
Sheep	acute*			
Goat	acute*			
Kangaroo	acute			
Dog	acute			Testis degen.*
Duckling	acute			
Turkey		Gizzard necrosis*		
Chick				
-embryo			Lethal ring, hemorrhage, and death	
-pullet			1. Exudative diathesis 2. Generalized edema 3. Encephalomalacia	
-adult	slight			Testis degen.*
Guppy fish	acute			Testis degen.*

* Not proved to be prevented by pure tocopherol.

unrecognized disturbances in adjacent or distant tissues. In other instances rather specific tissue changes occur, as in the seminiferous epithelium and often in the musculature, unassociated with any external symptoms or evidence of impaired growth or vigor. Intentionally left out of Table III, but discussed in subsequent sections, are other manifestations whose specificity is reasonably well established (retardation of body growth) or

doubtful (lesions of the nervous system, kidney lesions, endocrine dysfunctions).

It is natural to wonder whether, in view of the demonstrated changes in smooth musculature in certain forms, the vascular changes might be secondary to dysfunction of the musculature of the vascular wall or its nerve supply. There is at present no convincing evidence to substantiate such an explanation, or to indicate the alternate possibility that the muscle lesions are secondary to vascular changes. Since neither of these postulations would account satisfactorily for other dysfunctions which do not fall clearly into the categories of muscle or vascular lesions there are left open two major considerations: (1) that vitamin E is related to some single fundamental metabolic change which, to a variable degree, is essential to the well being of muscular, vascular, embryonic, and male germ cell tissue, or (2) that the vitamin functions in a variety of unrelated metabolic processes.

Assuming that the former explanation is the correct one, it is conceivable that in those forms where only acute dystrophy has been observed the metabolic needs of the striated musculature for vitamin E are so much greater than those of other tissues (*e.g.* smooth muscle, seminiferous epithelium) that the latter are still at least structurally unaffected at a level of vitamin E depletion which is incompatible with animal survival. On the other hand, tissues affected by lack of the vitamin in one species may in another species, through some peculiar and unrecognized difference in metabolic requirements, function adequately without the vitamin or in the presence of much smaller amounts of it. Such an explanation could account for the absence of testis injury and the minor muscle damage in mice fed diets as rigorously, or more rigorously, depleted of vitamin E than those producing the usual sequence of events in rats; and also for the differences in response of skeletal and gizzard musculature to E depletion in avian species.

Too little attention has been given to the possible effect of the presence or absence of dietary components other than vitamin E in promoting or precipitating the symptoms considered pathognomonic of the deficiency state. To be sure, the effect of cod liver oil in producing muscular dystrophy in herbivorous animals, even when the vitamin E content of the diets is otherwise adequate, has been extensively studied and generally attributed to the destruction of the vitamin by unsaturated fats either before or after ingestion. On the other hand, recent evidence that exudative diathesis and encephalomalacia can be accentuated or suppressed by dietary alterations which do not affect the vitamin E content (p. 139) suggests that certain non-vitamin components of the diet may affect the physiological state of the susceptible tissues in such a way as to make them either more

resistant or more sensitive to the deficiency of E. Probably in no other vitamin studies have such clear-cut phenomena of this type been revealed. While they afford no explanation for the varied symptomatology of experimental E deficiency, they do serve to emphasize the importance of regarding the latter not simply in terms of specie or tissue differences but also in terms of the reaction of cells and tissues subjected to varied metabolic demands in the absence of vitamin E.

It is perhaps fortunate that the experimental studies leading to the discovery of vitamin E were based upon rats. No other animal type has kaleidoscoped into its life so many of the gross and histopathological phenomena characterizing vitamin E deficiency in experimental animals. As will be noted (Table III) muscular dystrophy is the only lesion so far demonstrated in the majority of animals studied. It is interesting to speculate upon the developments which might have occurred, and the terminology which might have appeared in place of what now must be considered inadequate and misleading ("antisterility," "fertility," "reproductive"), had the guinea pig, hamster, or one of the avian species been the experimental animal on which the discovery of this new vitamin was based.

The following sections will give a brief resumé of those structural changes of the E deficiency state which appear to the writer to demand closest correlation with the ever growing knowledge of metabolic dysfunctions before our present crude concepts of vitamin E functions can be replaced by true understanding.

2. Sterility in the Male

Male rats severely depleted of vitamin E from early life show an irreversible degeneration of the seminiferous epithelium at the onset of sexual maturity, but no injury during adolescence. The process may be delayed in proportion to pre-weaning storage or post-weaning administration of E (157, 158). Opinions differ as to whether the alterations in the nuclear (147) or in the cytoplasmic (191) structure of the epithelial cells best reflect the deeply seated metabolic disturbances, which precede demonstrable histopathological changes to such an extent that administration of vitamin E as much as 10 to 15 days prior to the onset of the latter usually fails to prevent or retard the degenerative process (151). Within several weeks practically no germ cells remain in the seminiferous tubules. The cellular changes have been described in detail elsewhere (84, 147, 150). The ensuing sterility is truly permanent. While there exists evidence that some restoration of the seminiferous epithelium may occur after prolonged periods of therapy, neither restoration of fertility nor the presence of mature spermatozoa in the epididymis has been reported.

No testicular injury occurs in male mice reared for 400 days (37), or maintained through seven generations (93), on E-deficient diets which

readily induce first pregnancy resorptions in mice and rats and early testis degeneration in rats. This surprising finding suggests some unexplained difference in the metabolic demands of the seminiferous epithelium in these two closely related species. The striking sex difference in E requirements of mice has no parallel in rats (151). The male germ cells are unaffected in rabbits maintained in a prolonged state of chronic muscular dystrophy (135, 141) and in dystrophic guinea pigs (96). As mentioned before, this does not conclusively exclude the need of vitamin E for maintenance of germ cell integrity in these species whose musculature possesses such a high priority for the vitamin.*

Testicular degeneration resembling that seen in the rat, but not yet proven to be irreversible, occurs also in the chick (9, 10) fed diets of natural foods treated with ferric chloride to oxidize vitamin E present. Of particular significance are the recent observations (45, 46) that the male guppy fish (*Lebistes reticulatus*) reared on synthetic as well as iron-treated diets reveals a suppression of testicular development and similar degenerative changes in the mature germinal epithelium, together with dystrophy of the striated musculature and unusual cell changes in certain visceral organs; these changes, which are much more difficult to induce in sexually mature fish, are preventable by pure α -tocopherol. Preliminary tests suggested an irreversible type of testis injury; the response of other tissues was not determined. On the other hand, tadpoles reared on the same diets fail to metamorphose but show no suppression or structural alteration of the gonads of either sex (188).

3. Sterility in the Female

a) *Mortality of the Fetus.* In female rats deprived of vitamin E the processes of estrus, ovulation, conception, and implantation of fertilized ova are entirely normal. However, the developing embryos and fetal membranes show abnormalities of development resulting in intra-uterine death followed by rapid autolysis and resorption of the products of conception (84). Administration of sufficient vitamin E as late as the 10th day will usually prevent the resorptive process. Repeated resorptions do not affect sexual functions or the ability to complete pregnancy if sufficient vitamin is supplied, although prolonged depletion of E is associated with decreased fecundity and increased requirements of vitamin E for the completion of gestation (75, 76).

When sterile rats are given border-line doses of vitamin E, as used in routine bio-assay tests, the implantation sites at any stage of pregnancy may exhibit all gradations between early resorption and viable fetuses.

* Since the preparation of this manuscript Pappenheimer and Schogoleff, *Am. J. Path.* 20, 239 (1944) has reported the occurrence of testis degeneration in guinea pigs subjected to chronic vitamin E deficiency.

Fetuses which succumb during the last few days of pregnancy are usually delivered dead; those which are viable at delivery rarely survive more than a few days unless they, or the mother, are given an additional supplement of vitamin E. Efforts made to utilize this gradation in intensity of biological response as a criterion for bio-assay have not given the results first anticipated (149, 152).

The prolongation of gestation reported by Barrie (22) in rats given critically low doses of vitamin E must be attributed to experimental factors other than lack of E. Countless other investigations have demonstrated the almost invariable occurrence of parturition between the 21st and 23rd days of pregnancy. An extensive series of rats fed border-line doses of E and laparotomized during the last week of gestation have shown no abnormalities of parturition (151, 152). Barrie's suggestion that resorption of some fetuses delays development in others and thus prolongs gestation, possibly through deficiency of anterior pituitary hormones, is not in accord with the observation that one or two fetuses surviving at laparotomy in late pregnancy may be delivered, dead or living, at normal term (152).

The cause of fetal death and resorption *in utero* has been generally attributed to a suppressed development of the mesodermal derivatives of the embryo, especially those of the hematopoietic tissues—blood islands of the yolk sac and embryonic liver (84, 215). More recent studies (154, 155), based upon examination of later (16-day-old) fetuses, have revealed no serious depression of hematopoietic function in the low E embryo but indicate that abnormalities of the vascular system, characterized by stasis, distention, and thrombosis (especially of venous channels) which lead eventually to general ischemia and sometimes to frank hemorrhage constitute the real causes of fetal death. These vascular changes, the incidence of which is indirectly proportional to the level of critical vitamin E dosage, are not influenced by separate administration of vitamin C or vitamin K. They have not yet been demonstrated in the mouse, in which the process of fetal resorption otherwise resembles that in the rat (37, 93). These changes bear certain resemblances to the capillary thrombi of nutritional encephalomalacia (185, 186, 227), the exudative diathesis (54) of vitamin E-deficient chicks, and the vascular lesions in the early chick embryo hatched from eggs containing inadequate vitamin E (1, 6). Discovery of the fundamental cause of any one of these phenomena should open the way to a better understanding of the others.

b) *Endocrine Relationships*. It was perhaps natural that the character of the reproductive impairment in the two sexes of the vitamin E-deficient rat should suggest some endocrine disturbance. During the past fifteen years there has appeared a considerable literature relating the sterility of E deficiency to dysfunction of the anterior pituitary, or secondarily to

hyperplasia of the thyroid, and ascribing to concentrates of vitamin E physiological properties resembling those of the gonadotropic and estrogenic hormones. Unfortunately, the pure tocopherols were not available when many of these studies were conducted. This subject has been extensively discussed in a separate review (150) to which the reader is also referred for a more detailed description of the reproductive system in vitamin E deficiency. Subsequent reports have served only to confirm the conclusions that gonadal-hypophyseal dysfunctions which may exist in the vitamin E deficient rat are more justifiably considered to be the result of, rather than the cause of, the reproductive disturbances characteristic of the deficiency state.

Synthetic *d,l*- α -tocopherol acetate, administered orally or parenterally, has no estrogenic effect on immature female mice (19) or rats (66) and does not alter the activity of the gonads, vagina, or uterus of immature hypophysectomized adult rats (66). Furthermore, E deficiency sterility in rats cannot be prevented by administration of sex or gonadotropic hormones (33, 66).

The careful studies of Biddulph and Meyer (27) offer convincing evidence that the structural state and hormonal activity of the endocrine system of female E-deficient rats are normal, and that certain changes occurring in the pituitary, adrenal, and thymus of E-deficient males are secondary phenomena rather than specific effects of E deficiency. Other reports (86, 129) concerning loss of sex interest and cytological changes in the accessory sex glands and anterior pituitary in rats fed E deficient diets supplemented with considerably more than the minimal requirement of wheat germ oil or α -tocopherol for preventing testis damage, although vaguely interpreted as evidence of injury to the interstitial tissue of the testis, appear rather to emphasize the possibility that other unrecognized factors in the diet (such as rancid fats) or the experimental environment may afford a better explanation for certain endocrine and other disturbances otherwise attributed to lack of vitamin E.

An example of the latter type of error merits particular attention. There have appeared numerous reports attempting to relate the reproductive disturbances of E deficiency to thyroid dysfunction, secondary to altered production of thyrotropic hormone by the anterior pituitary (21, 202, and others), and to ovarian dysfunctions (35). Numerous other investigators have been unable to confirm these findings. The demonstration (28) that a deficiency of iodine in the E deficient diets or in the drinking water produces alterations in the thyroid similar to those ascribed to E deficiency seems to explain the controversial findings.

Clinical literature contains numerous postulations concerning synergistic relationships between vitamin E and the sex and gonadotropic hormones.

The experimental and clinical evidence on which they rest does not appear sufficiently trustworthy or concordant to warrant their incorporation in the present discussion. In the past there has been a tendency for investigators of this general problem to ignore the repeatedly demonstrated fact that the reproductive damage observed in E deficient rats, in its various expressions, is decidedly different from that due to hypophyseal insufficiency or to multiple endocrine dysfunctions. Postulations that the metabolic disturbances in nutritional muscular dystrophy may have their genesis in pituitary and sex gland dysfunction (216, 217) are not convincing, and have not been substantiated by direct experimental tests (168). Certainly the great preponderance of experimental evidence exonerates the anterior pituitary and thyroid as instigators of reproductive and other disturbances of vitamin E deficiency.

c) *Veterinary and Clinical Implications.* Because of the difficulties of experimental approach, evidence concerning the reproductive and other needs of vitamin E for man and domestic animals is largely circumstantial. It is not the purpose of this review to discuss the extensive literature on this subject, certain phases of which have been summarized elsewhere (200, 221, 222). The early waves of enthusiasm over the therapeutic value of vitamin E in treating sterility and abortion encountered in veterinary and clinical medicine, and subsequent waves centered around its use in clinical myopathies and neuropathies as well as in primary fibrositis (interstitial myositis), have now become little more than ground swells. It is hoped, however, that each wave has deposited on the shores some observations of real merit for continued advance. The difficulties in statistical treatment of results in the face of inadequate control material (145), the problem of properly evaluating the effect of other therapeutic measures instigated in conjunction with E therapy, the extensive use of wheat germ oil or concentrates of the latter rather than the pure vitamin in earlier studies, and the difficulties of explaining an assumed deficiency state in the face of presumably adequate diets, all contribute to the perplexity of the problem and the difficulty of evaluating the clinical usefulness of the vitamin.

Biological determinations of the vitamin E content of a wide variety of common cattle foods, many of them of such poor quality as to not support milk production, have not offered a very promising approach to the experimental production of vitamin E deficiency in cows (179); unless it be found that their E requirements are relatively much greater than those of the rat. Bio-assays of cereal grains and grasses indicate that the daily intake of α -tocopherol for a 1000 pound cow on summer pasture would be the equivalent of that contained in 4-7 pounds of good wheat germ oil (39). Such observations as these mitigate against the reputed therapeutic

value of infinitely smaller doses of the oil in cattle as reported by Vogt-Moller (221, 222) and others.

The evidence that vitamin E is beneficial in treating habitual abortion, threatened abortion, premature separation of the placenta, and certain forms of toxemia of pregnancy is contradictory and little more than suggestive at the present time. The observations and statistical analyses of Malpas (145), indicating that the results reported up to 1939 were not significantly different from what might have been expected with no treatment whatever, have been re-analyzed by Bacharach (15) in the light of other clinical data and the conclusion reached that there seems "to be little doubt that sources of vitamin E had a direct effect in making possible full-term births among women who had had two or more consecutive abortions or miscarriages."

The postulation of Shute (199) that the blood serum of E deficient rats and that of aborting women presumably deficient in vitamin E shows an increased content of estrogenic factors, which inhibits continued invasion of the maternal tissues by the fetal villi unless held in equilibrium by administered vitamin E, represents an interesting speculation. In this respect vitamin E is considered to have anti-estrogenic properties similar to those of the corpus luteum hormone. The reliability of the antiproteolytic test on which this hypothesis is based has been questioned by Cuthbertson and Drummond (48) and Shute's response (201) is not convincing.

Obviously much more well controlled evidence, based upon large therapeutic doses of pure tocopherols and application of improved chemical methods for their detection in blood serum, is needed before it can be definitely established that habitual abortion or other dysfunctions of reproduction in the human female are attributable to lack of vitamin E.

4. Nutritional Muscular Dystrophy

a) *Nature of the Lesions.* The relation of vitamin E to the nutritional muscular dystrophy first described by Goettsch and Pappenheimer (96) in guinea pigs and rabbits was at first uncertain, since preparations of wheat germ oil used failed to prevent the disorder. For eight years attention was focused upon the complicity of an additional water-soluble factor, and upon the toxic effects of dietary cod liver oil. The literature on this question is ably reviewed by Mackenzie and McCollum, *et al.* (136, 139, 140), whose careful and exhaustive studies first demonstrated that vitamin E is the sole limiting factor in this dietary disease. It has since been revealed that necrosis of striated musculature constitutes the most common manifestation of vitamin E deficiency in vertebrate species and, in many instances, the only lesion so far observed (Table III).

The histopathological changes observed are essentially those well known

to the human pathologist as hyaline, waxy, or Zenker's degeneration, usually associated with acute infections and fever. They are characterized by loss of striations, multiplication and irregular distribution of sarcolemma nuclei, and swelling of the sarcoplasm which in turn becomes structureless and vacuolated. The whole, or merely parts, of irregularly scattered fibres are affected. Edema and inflammatory reactions in the interstitial connective tissues, as well as calcification of necrotic muscle fibres, may occur. Reparative changes from intact regions of the sarcolemma, as well as complete atrophy and replacement by connective tissue, occur simultaneously and increase the difficulties of interpretation. Until the nature of the underlying metabolic disturbance of the muscle cells is better understood variations in the histological picture in different species, and even in the same species at different age periods (as in the rat), are of minor importance.

The literature on the subject of nutritional muscular dystrophy has been comprehensively surveyed in several excellent reviews (83, 181, 184), that of Pappenheimer (184) being the most complete and recent. A few general observations which appear to have particular bearing upon the metabolic problems discussed later warrant special note.

The acute type of dystrophy observed in the striated musculature of young rats (174, 180) and ducklings (186) and in the gizzard musculature of turkeys (186): (1) has been produced experimentally only during the period of early adolescence and rapid growth; (2) frequently shows spontaneous remissions of symptoms with rapid repair of the lesions; (3) shows poor response to therapy; and (4) often results in sudden death. The same is generally true of exudative diathesis and encephalomalacia in chicks (54, 186). The dystrophy in guinea pigs and rabbits, on the other hand, (1) is easily produced in mature animals; (2) is gradual in onset; (3) rarely shows spontaneous remission; (4) shows more histological repair concomitant with the degenerative process than does the acute type; and (5) responds to therapy by structural and metabolic repair unless advanced stages have been reached. This response of dystrophic rabbit muscles to E therapy, as reflected by the creatine excretion of the animal, has been suggested as a semi-quantitative method of bio-assay of the vitamin (136, 140).

The muscle lesions in adult rats, whether they concern the skeletal or smooth muscle, (1) are very gradual and progressive in development; (2) cannot be repaired, but may be arrested at any stage, by vitamin E therapy; (3) are never the primary cause of death of the animal; and (4) show relatively little histological evidence of repair in association with degenerative changes. The lesions in mice, while producing no external symptoms and involving relatively small numbers of muscle fibres, are most marked

during the prepubertal period (183); a phenomenon also common to low-E rats.

Muscular dystrophy has been produced in goats and sheep (59, 144) and accidentally in the tree kangaroo (101) only with natural diets containing considerable amounts of cod liver oil or other fish oils. The guinea pig and rabbit also become dystrophic on such diets (59, 144), as well as on low-fat diets devoid of cod liver oil (139, 143) and iron-treated diets of natural foods (96). Failure of the latter type of diet to produce muscle lesions or reproductive disturbances in goats (212) has not received satisfactory explanation. The finding of typical muscle lesions in a cold blooded vertebrate, the guppy fish (45, 46), reared on iron-treated and on purified diets, and their prevention by α -tocopherol, signifies a rather universal need for vitamin E in the maintenance of muscle structure and function.

The peculiar dystrophy-inducing action of cod liver oil in herbivorous animals, considered in earlier studies to represent a toxic effect (59, 144), is now attributed to the presence of unsaturated fatty acids which promote the oxidative destruction of vitamin E either in the diet or in the digestive tract (139, 161). The possibility that the absorption of the vitamin, or its utilization by tissues after absorption in the intestine, may be interfered with is considered unlikely. Doses of tocopherol which are curative when fed alternately with cod liver oil at 24-hour intervals (198) lose their effectiveness when both are administered together (139). Furthermore, prolonging the induction of the oil by hydrogenation (162) or addition of a stabilizer to reduce the rate of autoxidation (161) abolishes the dystrophy-promoting action of the oil.

b) *Relation to the Nervous System.* The biochemical changes occurring in muscles during the onset and cure of dystrophy, as outlined earlier, are not at all suggestive of a neurogenic origin of the muscle lesions, such as proposed by Ekblad and Wohlfart (69) on the basis of nerve cell atrophy in the spinal cord of dystrophic guinea pigs. The latter changes are non-specific and occur under various experimental conditions. There also exists general concordance that no significant changes occur in the spinal cord, peripheral nerves, or motor end-plates in dystrophic guinea pigs (43, 96, 193), rabbits (96), rats (180), or mice (183). A temporary decrease in motor end-plates which may occur in young rats (211) is regarded as secondary to the muscle damage. Physiological studies of the contractile power of dystrophic muscle (126) and the protection afforded by denervation and by tendon section (181) further emphasize the myogenic origin of the lesions.

Retention of permanent paralysis in some rats surviving advanced late-lactation paralysis (63, 85), motor incoordination observed in adult rats after prolonged deficiency (138), and reported lesions in the spinal cord

and higher centers of young (133) and old (68, 163, 164) paralyzed rats, have also indicated a specific involvement of the nervous system. On the other hand, others have reported negative findings, and a searching re-examination of the nervous system by Wolf and Pappenheimer (228) has brought forth quite convincing evidence that the lesions previously described are due to some factor in the experimental procedure other than the lack of vitamin E.

These observations are of particular significance in view of the fact that the lesions in the central nervous system of E deficient rats described by Einarson and Ringsted (68) were primarily responsible for the intense interest and hope aroused several years ago in the clinical use of vitamin E for treating amyotrophic lateral sclerosis and other chronic degenerations of the nervous system. Since 1940 no less than thirty clinical reports have appeared. In general the more recent the report and the greater the doses of E given, even in the form of pure α -tocopherol, the more discouraging have been the results (230). The actions of sulfathiazole in precipitating a syndrome resembling amyotrophic lateral sclerosis in man (225), and of sulfasuccidine in inducing muscle lesions in rats (50), both types of injury being preventable by vitamin E, are of interest. Efforts to produce a state of vitamin E deficiency in monkeys have so far proved fruitless (156). Although no clinical myopathy or neuropathy yet explored has proved to be a counterpart of the nutritional muscular dystrophy of laboratory animals, and although the reputed efficacy of α -tocopherol in the treatment of primary fibrositis (209, 210) has received no confirmation, hope still exists that vitamin E will eventually find a valuable application to clinical medicine.

c) *Metabolic Disturbances.* The concomitant occurrence of degeneration, repair, calcification, and fibrosis in damaged muscles interferes greatly with the proper interpretation of chemical analyses of the tissues. Changes in the electrolyte pattern, involving a striking increase in sodium and chloride with corresponding decreases in potassium, phosphorus, and magnesium (as well as creatine), point to a loss of cellular elements from disintegrating muscle fibres whose contents come to resemble the interstitial fluids in composition (89) or become replaced by fibrous connective tissue (167). These interpretations are in close accord with the histological findings.

Observations on total phosphorus and phosphorus compounds (95, 134, 169) have been conflicting and have given no specific information concerning the metabolic disturbance. There occurs a significant increase in cholesterol and total lipids (104, 169, 171). An increased proportion of cholesterol esters, suggestive of disturbed cholesterol metabolism in the muscle, seems to be a characteristic feature (171). There is also a

definite lowering of muscle glycogen (34, 95). A detailed study of the metabolic picture during the onset and cure of dystrophy in the intact rabbit has been presented by Morgulis and Spencer (170).

Striking losses of muscle creatine and an associated creatinuria, proportional to the extent of muscle damage, are constant findings. The white muscles are more extensively altered than red muscles (94). The progressive development of the disease and the results of vitamin E therapy are readily detected by frequent measurements of the urinary creatine (140) or the ratio of creatine to creatinine in the urine (81). As many as six successive attacks of dystrophy have been precipitated and cured in individual rabbits (135).

Particularly noteworthy is the rapidity with which the chemical changes in the muscles are restored to normal following vitamin E therapy. These alterations are to be regarded as sequential to some underlying metabolic dysfunction of the muscle cells which, though as yet imperfectly understood, can also be recognized and measured by changes in their O_2 consumption. Investigation of the latter phenomenon, which has been recognized for some years but only recently given intensive study in the laboratories of Mattill and of Pappenheimer, has yielded highly interesting results which appear to open the way for future researches of even greater significance.

There occurs a significant but sometimes variable increase in the O_2 consumption in the dystrophic muscle of rabbits (91, 143, 218), guinea pigs (143), adolescent rats (125), older rats (110), and hamsters (108). Since this increased O_2 uptake occurs even in muscles of young rats and chicks which, though deficient in vitamin E, show little or no evidence of anatomical change, it can be attributed to changes in the metabolic activity of the muscle fibres themselves and not to proliferative activities of connective tissue cells which later invade and replace the damaged muscle tissue (125). Liver of low E rats shows no altered O_2 uptake, suggesting that the latter may be characteristic only of muscle tissue (125). The reported absence of such changes in duck muscle (218) may possibly be due to the fact that the muscles examined were already so extensively damaged that a true picture could not be obtained. The oxidative changes are highest during the more acute stage of the disease and may become negligible as symptoms or lesions become far advanced. They bear no specific relationship to species differences in normal oxygen uptake (110), the latter being greatest in rats and lowest in rabbits. The important studies of Kaunitz and Pappenheimer (124) have shown that changes in the total O_2 consumption of the intact rat parallel those observed in isolated muscles, and that inanition in the deficient animals has no significant effect on the oxidative changes.

The hamster, which Houchin and Mattill (108, 110, 111) have found to be particularly susceptible to muscular dystrophy, shows an unusually high

increase in O_2 consumption (240–250% of the normal), which is reduced toward normal within 27 hours after oral administration of α -tocopherol acetate (108). It has been repeatedly shown that tocopherol and its acetate are relatively ineffective when administered subcutaneously, intramuscularly, or parenterally. Even after oral feeding there is considerable delay involved in absorption, transfer, and perhaps phosphorylation before tocopherol becomes available to muscle cells. The slightly water-soluble ester, α -tocopherol phosphate, becomes readily available to dystrophic tissues when injected (81, 142). Intravenous injection of the phosphate compound reduces the high O_2 consumption of dystrophic rabbit muscle to normal in about 4 hours (112); when added to the substrate in *in vitro* experiments it immediately restores the O_2 consumption of rabbit and hamster muscle to normal. Restoration of normal creatine, involving a temporary early reduction not observed after administration of more slowly acting tocopherol compounds, occurs 10 or more hours later; while chloride restoration is more delayed (112). Tocopherol does not affect the O_2 uptake of muscles which already contain optimal amounts (109, 111).

The observations just cited clearly indicate that vitamin E has an important function in the control and regulation of oxidative processes in muscle cells. The increased O_2 uptake is characteristic only of intact dystrophic muscle, since it is lost after mincing or homogenation (109). The latter observations, suggesting an enzyme system possessing one or more water-soluble components capable of dispersing and reducing the concentration of the system, led Houchin (109) to investigate the succinoxidase system of dystrophic muscle. That of the hamster proved to have a succinoxidase activity 162% above normal and proportional to the degree of dystrophy, that of paralyzed suckling rats was somewhat less marked. This activity was diminished by α -tocopherol phosphate but not by α -tocopherol alone or in combination with desoxycholic acid. This suggests, as have other observations, that the tocopherols require phosphorylation in the body before they become active (109, 111).

While admitting that any concept of the mechanism of action of vitamin E still remains highly speculative, Houchin (109) makes the interesting suggestion that tocopherols, probably in phosphorylated form and acting through some known or perhaps as yet unknown enzyme system or systems, serve to inhibit or regulate oxidative processes of skeletal muscle. In their absence these mechanisms get out of control and allow the muscle to be consumed by its own fire. One naturally wonders whether these new developments in the vitamin E field have been timed to converge with some seemingly unrelated discoveries in the field of enzyme chemistry to reveal hitherto hidden mechanisms of cellular oxidation.

5. *Changes in Smooth Muscle*

The fact that lesions of the involuntary musculature have been encountered only in the young turkey and adult rat does not lessen their importance. The acute necrosis occurring in the turkey gizzard (118) bears many resemblances to the striated muscle lesions in other experimental animals. They differ markedly from the changes observed in the rat uterus (146). The latter, which are very gradual in their development and never fatal, are characterized by a faint yellowish discoloration which becomes apparent at the 2nd or 3rd months of E depletion and progressively increases to a dark muddy brown after 6 to 12 months. A similar discoloration, though much less intense, occurs in the oviducts, ovarian ligaments, retroperitoneal lymph nodes, seminal vesicles, and testes. Musculature of the urinary bladder and gastrointestinal tract shows no alteration.

According to Martin and Moore (146), the sarcoplasm of uterine smooth muscle cells reveals perinuclear accumulations of small yellow granules, whose number is proportional to the discoloration of the organ. Many cells eventually become engorged with granules, greatly enlarged, and distorted in shape; considered indicative of a slow, progressive degeneration. The granules contain no iron and are resistant to strong acids and alkali. Both tocopherol and concentrates of wheat germ oil prevent their appearance but cannot effect their removal. These findings have received general confirmation from many sources (23, 62, 75, 105, 156). Pale discoloration of dystrophic striated musculature has been frequently noted in the past but no particular significance attached to it. The observation (146) that this is due to an accumulation of brownish granules, similar to but less extensive than that occurring in smooth muscle cells, suggests a common structural pattern in response of muscle cells to lack of E having greater significance than the degenerative necrosis previously discussed.

A recent re-investigation of the muscle injury in long-term E deficient rats (156) has brought out certain additional points which deserve mention. Many of the enlarged misshapen granule-laden cells interpreted by Martin and Moore as degenerating muscle cells, but not mentioned in other cytological studies of the rat uterus (62, 105), appear to be phagocytic cells of the connective tissues which have become loaded with the pigment granules; the latter probably representing a metabolite which the muscle cell, lacking the ability to properly utilize or break down in the absence of vitamin E, liberates for the most part without losing its own integrity. It is of interest that similar phagocytic cells containing brownish yellow pigment have been observed in association with brain lesions in E deficient chicks (227).

Migration of these phagocytic cells by lymphatics from their site of for-

mation in the musculature (smooth and striated) may possibly account for their presence in the lymph nodes, spleen, ovary, and interstitial connective tissue of the testis. The yellowish pigment material, especially that in the phagocytic cells, possesses a remarkable affinity for basic fuchsin, is remarkably inert, and gives no reactions for hemosiderin, porphyrin, or lipids. Such large concentrations as exist in the rat uterus represent a challenge to the organic chemist.

Why the smooth musculature of the uterus should be so markedly affected, that of the seminal vesicle in the male much less involved, and that of the gastro-intestinal tract and urinary bladder so immune, is as inexplicable as the absence of any smooth muscle changes in severely dystrophic guinea pigs and rabbits (96, 181). It is possible that the survival period of the latter animals is too short to induce such changes, which naturally infers a much lower vitamin E requirement for smooth than for striated muscle—a condition certainly not characteristic of the female rat. The uterine changes occur in deficient rats castrated early in life (156) and in virgin rats (23, 146). The observations that the degree of discoloration is accentuated by pregnancy resorptions (23), unaffected by high E therapy for prolonged periods (146, 156), but diminished when pregnancies intervene during therapy (75) suggest some relationship to the metabolic state of the uterus. The unusual abundance of macrophages in the uterus in comparison with other organs, as revealed by vital staining (40), may have a bearing upon its intense discoloration after E deficiency. One would also expect their removal via lymphatics and vascular channels to be expedited by the physiological changes incident to the state of pregnancy.

Although discolored uteri show normal contractile responses to para- and sympathomimetic drugs (61, 105, 156), there occurs an increase in vitamin E requirements for successful gestations after extended periods of E depletion (23, 75). This has been ascribed to decreased ability of the placenta to transfer the vitamin to the embryos or general failure of the mechanism responsible for implantation of the ovum (75). The latter are primarily functions of the non-muscular portions of the organ. The part which the muscle injury may play in modifying the vitamin E requirements for pregnancy is not clear. The growth retardation observed in such animals appears not to be sufficient to significantly interfere with reproductive success, but may play an important part in the delayed sexual maturity and reduced fecundity of females observed in successive generations of rats on low E diets (76).

The cardiac musculature of E deficient rabbits is reported to be histologically normal (96), even after repeated attacks and cures of muscular dystrophy (135), and to show no alterations in cholesterol or lipid phosphorus (169). Some doubt exists (181) that the heart lesions observed by

Madsen (143) in rabbits are referable to deficiency of E. Although other investigators have failed to find myocardial lesions in young (174, 180) and adult dystrophic rats, the writer (156) has frequently observed in the heart muscle of rats subjected to prolonged E depletion lesions resembling those seen in the striated musculature.

6. *Dysfunction of the Vascular System*

The lesions found in vitamin E deficient chicks, while showing considerable variation among themselves, appear to be the reflection of some fundamental dysfunction of the vascular system. Their only possible counterpart in mammals is the hemorrhagic state recently observed in the rat fetus (p. 126).

a) *Embryonic Mortality in Chicks.* Unless the chick egg at the time of laying contains an adequate amount of vitamin E, embryonic death occurs at about the fourth day of incubation, due to early disintegration of the blood vessels of the blastoderm, frequent hemorrhage into the coelom and exocoelom, and formation of a dense ridge-like lethal ring in the blastoderm which interrupts the vitelline circulation. Embryos which survive this critical period of development later show spontaneous rupture of vascular channels within the embryo, usually associated with clusters of a characteristic histiocytic type of cell at the point of extravasation (1). The latter phenomenon has been observed in embryos used for routine class work in embryology (6). The incidence of first-week mortality bears a close relationship to the vitamin E content of the pullets' diet (20).

Day-old chicks reared on purified E deficient diets usually exhibit a state of exudative diathesis (53, 54) or of nutritional encephalomalacia (185, 186) or sometimes both, during the first month or so of life. The two symptoms possess certain features in common which warrant particular consideration: (1) their relation to dysfunction of the capillary bed; (2) their frequent spontaneous regression without change in the dietary regimen; and (3) the manner in which their occurrence can be influenced by changes in the diet which in no way affect its content of vitamin E.

b) *Exudative Diathesis.* This symptom is characterized by the accumulation of a plasma-like fluid in localized areas of the subcutaneous tissues, muscles, adipose tissues, and connective tissues generally. Under certain conditions fluid accumulations frequently appear in the pericardial and peritoneal cavities (30). According to Dam and Glavind (53, 54) these exudative effusions have essentially the same chemical composition as normal blood plasma, and frequently possess a greenish tinge due to decomposed hemoglobin. The affected tissues usually show edema, hyperemia, and increased permeability of the capillaries (as measured by the ability of the tissues to absorb trypan blue injected intravenously). No

significant changes have been noted in serum proteins, cell content, prothrombin content, or clotting time of the circulating blood. At the site of lesions undergoing spontaneous regression, the adipose tissue retains a buff color for some time. In severe cases there may occur degeneration of leg or breast muscles at the site of earlier lesions leading to permanent invalidism, or a perforating ulcer of the gizzard which is unrelated to the lack of vitamin E (52).

c) *Nutritional Encephalomalacia*. Nutritional encephalomalacia is characterized by the sudden occurrence of ataxia, head retraction and prostration associated with localized areas of edema, small hemorrhages, capillary thrombosis, necrosis of neural elements, and degenerative softening grossly visible in the cerebellum (185, 186). Histologically, there is enlargement of the smaller blood vessels, edema, proliferation of the capillary endothelium, small hemorrhages, and hyaline thrombosis of many capillaries, particularly at the Purkinje cell level of the cerebellar cortex where the blood vessels make a sharp right angle turn; all are indicative of circulatory disturbances as the primary cause of the lesions (227). There is no evidence that prolonged vasoconstriction or vasomotor paralysis can explain the hyaline capillary thrombi which form such a characteristic feature of the lesions. Of interest, in this connection, is the report (31) that the tissues of E deficient rats show a low cholinesterase content which is readily restored by α -tocopherol feeding.

During spontaneous or induced recovery, active growth and mitosis in endothelial cells of capillaries in the area of the lesions is a prominent feature (186). The occurrence of phagocytes containing brownish-yellow pigment near the site of old lesions calls to mind the buff color of tissues observed at the site of old exudative lesions referred to above, and the pigment accumulations in liver cells of chicks (5). Although such cells do give an iron reaction (186) they otherwise bear resemblance to phagocytic cells seen in the uterus of the rat. The absence of significant alterations in the cell plasma ratio, plasma volume, or blood volume (186) also suggests some functional alterations of the capillary wall, the same common denominator to which the lesion in exudative diathesis has been reduced.

Although muscle lesions rarely if ever occur in the E deficient chick (186), the pectoral muscles at least show a definitely increased O_2 consumption (124), the significance of which is not yet understood. Contrary to the findings of others, Adamstone reports that natural diets treated with ferric chloride will produce not only nutritional encephalomalacia (4) but also a series of other phenomena—lymphoblastomata (3), erythrophagocytosis and anaemia (5), intestinal ulcerations and sarcomas (7)—in chicks, depending upon the type of fish liver oil incorporated in the diet. Objection

again must be raised on the ground that vitamin E is not always destroyed by such treatment of diets (60) and that other undesired changes may occur.

d) *Dietary Factors Influencing Symptomatology.* The recent studies of Dam and his collaborators (51, 52, 55, 57) portraying the manner in which the rate of onset and general incidence of symptoms in the chick can be influenced by modifications of the experimental diet which are quite unrelated to its vitamin E content, have opened a fascinating story. It has been found that the symptoms of exudative diathesis are strikingly accentuated by the following procedures: (1) increasing the concentration of soluble salts, which may either disturb the osmotic equilibrium of the body tissues and fluids or increase autoxidation of fats in the diet; this also tends to favor production of pericardial and peritoneal exudates; (2) addition of histamine, when fat is also present in the diet, or addition of cholesterol, fat, or unsaturated fatty acids; all of which may contribute to a weakening of the capillary wall; (3) increasing carbohydrates and decreasing proteins, possibly due to tendency of high carbohydrate diets to enhance retention of tissue fluids. There also appears to be a definite protein-carbohydrate ratio which is optimal for the appearance of the exudates. Addition of lipocaic (a water soluble preparation from pancreas) or inositol, both lipotropic substances, affords protection against exudates even when the concentration of soluble salts is high; whereas choline chloride and gum arabic are ineffective.

On the other hand, qualitative and quantitative differences in the fat content of the diet exert the most marked influence on the rate and time of occurrence of the exudates; the same is also true of the symptoms of encephalomalacia referred to later. Exudates rarely occur if the diet contains only traces of fat, but develop to approximately the same degree with diets containing about 5% of cod liver oil, fatty acids from an equivalent amount of cod liver oil or linseed oil, 5% lard, or 5% commercial unsaturated fatty acids. Fresh and partly rancid cod liver oil are equally effective, whereas thoroughly rancid oil is without effect. In short, unsaturated fatty acids in one form or another seem prerequisite to the appearance of exudative symptoms which, in turn, are considered cardinal signs of vitamin E deficiency in chicks and can be prevented by pure α -tocopherol.

Encephalomalacia also appears only with diets containing fat, and much depends upon the particular fat used. Hog liver fat favors the symptoms of encephalomalacia much more than cod liver oil; a high content of lard acts likewise, especially when the protein-carbohydrate ratio is high. Inositol reduces the incidence and severity of encephalomalacia occurring on high fat diets whereas lipocaic does not. Diets which produce exudative symptoms only can be so modified, by incorporating lard at a high level

and decreasing the carbohydrate correspondingly, as to produce a higher incidence of encephalomalacia than of exudates. Furthermore, with the fat content of the diet remaining constant, lowering the carbohydrate further by a corresponding increase in the protein component of the diet, permits encephalomalacia to appear as the dominating symptom. When added to diets with a high content of lard, cholesterol inhibits the appearance of encephalomalacia but does not affect the exudative diathesis.

At last there has been offered a satisfactory explanation for the predominance of encephalomalacia in the classic studies of Pappenheimer and his colleagues (185, 186, 227) in which diets high in lard were used, and the almost universal occurrence of exudative diathesis in those of Dam and his associates (51 to 57) using diets lower in fat and high in carbohydrate. Whatever the proper explanation may be, it is obvious that a variety of dietary additions or omissions, exclusive of vitamin E, can alter within wide limits the manner of response of organs and tissues suffering from a concomitant deficiency of vitamin E. Admitting the dominant rôle of fats in the genesis of vitamin E deficiency symptoms in the chick, consideration must also be given to the possible effects exerted by other components of the diet, either through their influence on the autoxidation of dietary fats or their ability to directly alter the sensitivity of affected tissues. The action of inorganic salts as prooxidants, and of yeast as an antioxidant (44), might be cited as an example of the first alternative; the production in rats of muscle lesions, preventable by α -tocopherol, following the dietary addition of succinyl sulfathiazole (50) affords an example of the second. Dam (52) is of the opinion that vitamin E may have a function in preventing the undesired accumulation in tissues of certain normal or abnormal products formed in the intermediary metabolism of fat or carbohydrate, but does not ignore the possibility that the vitamin might, in some unknown manner, increase the resistance of the tissues to such breakdown products of metabolism.

The manifestations of vitamin E deficiency in mammalian forms, especially in the rat, are not so extensively affected by qualitative and quantitative changes in the dietary components. It should be noted, however, that dietary fats have for many years played a prominent rôle in vitamin E studies with herbivorous animals. Differences in their nature and state in the diet has undoubtedly been the basis for discord in many experimental results reported; a situation made more complicated by the unfortunate habit of increasing rancidity in synthetic diets by prolonged exposure at room temperatures before feeding, and by the ferric chloride treatment of natural food diets. It has been generally accepted that autoxidative rancidity in fats causes the destruction of vitamin E either in the diet or in the digestive tract or both (47, 139, 161, 223). The possible destruction of

E after its actual absorption by the organism has not been lost sight of, but no experimental evidence has yet been given to support such a possibility. Various phases of the dietary fat problem are discussed more extensively in a recent review (38).

The remaining alternative, that unsaturated fatty acids or their breakdown products may induce a direct or toxic damage to certain tissues, a damage counteracted in some unknown manner by tocopherol, has been considered unlikely on the basis that diets exceedingly low in fat produce muscular dystrophy in rabbits (139) and paralysis in rats (137), and that cod liver oil loses its ability to inhibit the curative action of vitamin E on muscular dystrophy in rabbits if the two are fed on alternate days (139, 141, 198). The suggestion of Mattill (159) that the cecum of herbivorous animals offers greater opportunity for more rapid and prolonged autoxidative changes to occur than in rats and other omnivorous animals is of interest in this connection.

In striking contrast to rabbits, both rats (18, 156) and chicks (30) can effectively utilize vitamin E concentrates or α -tocopherol dissolved in cod liver oil. Furthermore, although fetal resorption (which may or may not be identical to that of E deficiency) occurs in mice fed heptaldehyde (41), this substance and other active pro-oxidants do not interrupt pregnancy in rats (42, 60). The latter findings are not in accord with reports that products of rancidity in fats when fed or injected induce, in rats receiving additional vitamin E, a sterility of both sexes simulating that of E deficiency (128, 131). In fact, Kudrjashov (131) claims to have obtained these results using the non-saponifiable fractions of fats extracted from E deficient rats, on the basis of which he postulates that the injuries characterizing the deficiency state are secondary to toxic effects arising through disorders of fat metabolism.

This discussion of interrelationships between vitamin E, dietary fats, and fat metabolism in the body has been introduced at this point to illustrate the present array of conflicting evidence and to emphasize the complexity of the problems remaining to be settled before the functions of vitamin E are fully clarified.

7. Growth Inhibition

Retardation or decline of body growth which often precedes or follows the onset of E deficiency symptoms in many species has been shown to be a characteristic manifestation of the deficiency state only in the rat. Except perhaps in rats which recover from advanced paralysis during late lactation, adolescent growth is normal; however, after 2-4 months on E deficient diets the growth of the rat reaches a plateau and remains relatively stationary for many months before slowly declining (74, 138, 173, 177). Since the growth

plateau precedes by some months the appearance of adult muscular dystrophy, and since administration of vitamin E at any period elicits a definite growth response but effects no restoration of the damaged musculature, the growth inhibition would seem to be another reflection of disturbed metabolism due to lack of E. It would be interesting to know how much of the growth change prior to and after vitamin E therapy is dependent upon fat deposition in the various tissues.

Dietary fat has a definite influence on the growth pattern, as it does in the case of E deficiency symptoms in other species, the reason for which is quite obscure. Increased dietary fat in E deficient diets accelerates adolescent growth (177), and elimination of added fats induces an earlier and more consistent growth plateau (102). The latter observation has been utilized in a new bio-assay procedure based upon increase in body weight of the female during gestation (102). Since sterility in both sexes can be produced and maintained at levels of E depletion which have little or no growth retarding effect, it must be assumed that the E requirements for growth are considerably less than those for preventing sterility. Body growth in the mouse is not influenced by lack of vitamin E (93) which is in keeping with its greater resistance to deprivation of the vitamin.

The few studies dealing with growth stimulating effects of vitamin E on tissue cultures, carried out before pure tocopherols became available, are not at all convincing; those dealing with tumor growth are more numerous; since they have also failed to implicate vitamin E in any significant manner, they warrant no more than passing comment.

IV. MINIMAL REQUIREMENTS

Data concerning the minimal requirements of vitamin E for preventing the symptoms and lesions characteristic of different laboratory animals are fragmentary, only roughly approximate, and permit only limited generalizations. The data available in the literature have been calculated in terms of milligrams of free tocopherol per kilogram of body weight and tabulated in table IV, primarily for the purpose of recording the efforts so far made to establish minimal requirements for different laboratory animals. The obvious value of such data, as an aid in determining the character and the unity or multiplicity of the functional rôle of vitamin E, is dependent upon further exploration of the problem.

It should be kept in mind that in many of the experimental studies designed to determine these requirements due consideration has not been given to the influence of certain qualitative differences in dietary components which may significantly modify the results obtained. There is evidence that vitamin E requirements for the rat are increased by the addition of fats to the deficient diet (102). In view of the rôle of dietary

tocopherol in stabilizing stored fats (19a, 38) it is possible that the procedure of inducing rancidity with the intent of destroying traces of E in experimental diets (85a) may inadvertently decrease the amount of dietary tocopherol available for preventing the pathognomonic signs of E deficiency and give higher than normal values for minimal requirements. It is also possible that fatty acid components of fats may, in some unknown manner, alter the susceptibility of tissues to deprivation of vitamin E, as has been suggested in the case of chicks (52). Requirements may also increase considerably with advancing age of the rat (75, 85a). In view of these

TABLE IV
Estimated Minimal Daily Requirement for Vitamin E

Animal	Symptom	Tocopherol Used	Estimated Minimal Requirement as Free Toco- pherol mg. per kg.	Refer- ences
Rat	Sterility (♀ and ♂)	α-	0.33-0.48	(151)
Rat	Sterility (♀)	α-acetate	0.6*	(85a)
Rat	Sterility (♂)	α-acetate	>1.0-<3.0*	(85a)
Rat	Uterine pigmentation	α-acetate	>1.0-<3.0*	(85a)
Rat	Uterine pigmentation	α-	>0.3-<1.0*	(165)
Rat	Early paralysis	α-	>1.3-<13*	(98)
Rat	Adult dystrophy	α-acetate	<1.6*	(127)
Rat	Adult dystrophy	α-acetate	<0.6*	(85a)
Rabbit	Muscle dystrophy	α-acetate	<0.7-1.0	(140)
Rabbit	Muscle dystrophy	α-acetate	0.2-0.4	(79)
Rabbit	Muscle dystrophy	α-acetate	0.32	(80)
Rabbit	Muscle dystrophy	α-phosphate	0.31	(81)
Chick	Encephalomalacia	α-	1.1	(186)
Chick	Encephalomalacia	α-	<7.5	(55a)
Chick	Exudative diathesis	α-acetate	<3.0*	(56)
Duck	Muscle dystrophy	α-	>4-<16*	(182)

* These values, which have been calculated by the writer and are influenced by necessary estimations of the mean body weight of the experimental animals for which few data are given in the original reports, should be regarded as approximations.

modifying factors, and the errors inherent in the calculations, it is surprising that the estimates presented in Table IV fall within such a limited range.

Recent observations concerning the influence of different fats and fractions of fats in retarding or precipitating the symptoms of E deficiency in chicks (52) emphasize the difficulties in arriving at a satisfactory evaluation of minimal requirements for these animals. The influence of cod liver oil in modifying the response of rabbits to E deficiency is well recognized and has been taken into consideration in estimating the minimal requirements for this species; which undoubtedly accounts for the close agreement shown.

The matter of differences in sex requirements in response to E deficiency, represented by numerous fragments of evidence reported in the literature, has a bearing upon the question of minimal requirements. Little mention has been made of sex differences in birds and herbivorous animals; the rat being the chief topic of such discussions. Recent efforts to establish anti-sterility requirements for the two sexes of the rat are not in accord; one study reporting no essential difference (151), another study indicating a considerably higher requirement for males than for females (85a). In the mouse, on the other hand, sterility is readily induced in females but never in males, even after prolonged E depletion (37, 93). In rats (126) and puppies (11), but not in mice (183), the male seems more susceptible to muscular dystrophy than the opposite sex. It would be of interest to know whether this same differential prevails after castration.

V. INTERRELATIONSHIPS WITH OTHER VITAMINS

Most vitamins belong to widely different groups of chemical compounds. The close chemical relationship between the tocopherols which are derivatives of hydroquinone and the vitamin K compounds which are derivatives of naphthoquinone, the similarity of the methods used in their synthesis, and the fact that their activity is possessed by many other closely and distantly related chemical compounds, constitute a unique situation. The relation of vitamins E and K to the vascular system and hemorrhagic states is interesting even though the types of dysfunction created by their absence are quite dissimilar. Despite their chemical similarity, it has not been found possible to transform tocopherols into compounds having vitamin K activity, and only one of the latter substances has been transformed into a tocopherol. This latter compound (naphtho-tocopherol) is reported to possess the biological activity of both vitamin E and vitamin K (213).

Considerable interest has centered around the action of vitamin E as a conservator of vitamin A in the animal body, as discussed more extensively in a recent review (106). This is based upon evidence that storage of vitamin A in the rat liver is significantly enhanced by feeding relatively small amounts of α -tocopherol (58, 107, 165) or somewhat larger doses of its acetate (16). The lesser activity is attributed to the fact that the acetate is relatively inactive in the stomach and intestine where this synergistic action is thought to occur (106, 196). This action, which is greatest when both vitamins are fed simultaneously (196), is related to the antioxidant activities of the tocopherols and their ability to protect vitamin A during its passage across the gut wall (107, 189). These findings are not only of great physiological interest but also have an important bearing upon the accuracy of biological assays of vitamin A.

There is some evidence that depletion of vitamin E stores in the male rat

are accelerated by elimination of vitamin A from the diet (132, 147), and that the antisterility action of tocopherol may be influenced by added β -carotene (107). Such observations are at least suggestive that this synergistic relationship may be a reversible one, to the extent that vitamin A may enhance the storage and utilization of vitamin E.

Hickman, *et al.* (106, 107) make the interesting suggestion that vitamin E may also protect vitamin A at its site of utilization by the tissues of the body, and go so far as to postulate that the disturbances in the fetus and musculature of the E deficient rat might be secondary to a lowering of vitamin A in the maternal blood serum or the musculature, respectively. This latter possibility seems quite unlikely in view of the dissimilarity in the E deficiency and A deficiency lesions in the reproductive system of male (147) and female rats (148), and the evidence that α -tocopherol can prevent the muscle lesions occurring in rats fed diets deficient in vitamin A and E but cannot modify the typical A deficiency lesions (130).

Other somewhat isolated observations relative to a synergistic relationship between vitamin E and other vitamins, as well as between E and certain hormones, are referred to by Hickman (106). Their value in interpreting the physiological rôle of vitamin E is still uncertain.

VI. THEORIES OF PHYSIOLOGICAL FUNCTIONS

The antioxidant function of the tocopherols, important as it may be, cannot represent the prime rôle of vitamin E in the animal body. The inability of other antioxidants to substitute for vitamin E, the inverse relationship between antioxidant and biological actions of α -, β - and γ -tocopherols, lack of evidence that tocopherols retain their antioxidant activities after their ingestion, and the manner in which changes unrelated to the phenolic hydroxyl group in the molecule alter biological activity of the tocopherols, all suggest a more fundamental and complex rôle in metabolic processes. It is not surprising that none of the various theories so far proposed to explain the physiological rôle of vitamin E has proven adequate. Although based upon restricted knowledge and molded by narrowed viewpoints, these theories do indicate types and trends of thinking which have evolved with our growing knowledge of vitamin E. For this reason, as well as for other virtues which they may possess, they warrant brief consideration.

About ten years ago, Adamstone, impressed by histopathological changes noted in the E deficient chick, postulated that vitamin E may exert a controlling influence over cell proliferation (2, 3). Although he has more recently proposed that vitamin E is related to the metabolism of cholesterol and other anthracene compounds (8), his own experimental findings appear to be more in accord with the earlier idea.

Of similar vintage, but of slightly different character, is the suggestion (147) that vitamin E may be necessary either for the synthesis of nuclear chromatin or for maintenance of the normal physico-chemical state of the latter, based largely upon the irrevocable nuclear changes in the rat testis. This concept also appears to be limited in perspective and applicability. However, without exactly bringing the skeleton out of the closet, brief reference will be made to a series of established facts and recent observations which, whether they be pertinent to this theory or not, may justify some consideration in any effort to appraise the physiological function of vitamin E.

Nucleoproteins, especially those of mature sperm, are characterized by an exceptional content of arginine. Recent evidence (197) indicates that a deficiency of this amino acid will induce in man a rapid reduction in sperm counts which can be restored by return to an adequate diet; and, in rats, a testis degeneration closely resembling that of vitamin E deficiency. Unfortunately the reparability of the latter process has not been determined. The retarded growth of arginine deficient rats, due to their limited capacity to synthesize this compound (as shown by work of Rose and his associates) also resembles that of E deficiency. On the other hand, the mouse, which apparently can synthesize its arginine needs (24), exhibits neither testis degeneration nor retardation of growth when deprived of vitamin E.

Although the metabolic relationship between arginine and creatine is not fully established, there is considerable evidence (26) that tissue arginine represents one of the important precursors of creatine. The possibility that a defective production or utilization of arginine may be involved in the metabolic dysfunctions of dystrophic muscles is purely hypothetical. Although the creatinuria of muscular dystrophy may merely be incidental to the breakdown of muscle protein, there is good evidence that vitamin E is intimately concerned with creatine metabolism (see H. H. Mitchell, in volume I of this series, p. 179). The suggestion (26) that the creatinuria of nutritional muscular dystrophy might be due to hydration of creatinine to creatine, due to increased water content of the tissues, is of interest; especially in view of the fact that alteration in the normal water balance of tissues is a consistent finding in the biochemical and histological examinations of tissues affected by E depletion. It is, of course, impossible with the data now at hand to clearly discriminate between those changes which represent the cause, and those which reflect the effect, of metabolic alterations.

As mentioned in an earlier section (p. 126) numerous attempts have been made to attribute the disturbances of vitamin E deficiency to dictates of dysfunctional endocrine glands (anterior pituitary, sex glands, or thyroid) or to synergistic relationships between the vitamin and hormone production.

Much of the evidence for and against these suggestions has been critically analyzed elsewhere (65, 150). On the whole the experimental evidence prompting these postulations is weak, and not always subject to confirmation. In fact, successive reports from the same laboratory have retracted previous theories and proposed new ones (33, 34, 35). Although it cannot be denied that hormone actions may play some rôle in modifying the manifestations of E deficiency, or be themselves affected by the latter states, there is no convincing evidence that they play a primary rôle in the genesis of these dysfunctions.

The interesting suggestion that vitamin E might represent an important link in oxidation-reduction mechanisms of cells (116, and others) has been abandoned because of the biological inactivity of the quinol-quinone oxidation products of α -tocopherol. There are suggestions that vitamin E may function, in some still obscure manner, in certain phases of fat or carbohydrate metabolism. The accumulation in the tissues of intermediary products of such metabolic processes offers a reasonable explanation for variable symptomatology in E deficient chicks (52). Certainly the lesions of E deficiency in various animals studied are influenced by the fat content of the diet to an extent which cannot be entirely related to oxidative destruction of dietary traces of the vitamin.

It remains for future studies to determine the extent to which existing and newer knowledge concerning the interrelationships between vitamin E and fats or their breakdown products can be correlated with the demonstrated participation (109-112) of the vitamin in the control of oxidative dysfunctions existing in dystrophic muscles. Failure of tocopherols to influence the oxidative processes in normal muscle may be all the more indicative that dystrophic muscle suffers from a distorted metabolism or abnormal accumulation of metabolites in the tissues, which is merely reflected in the increased oxidative activity. Until more is learned concerning the enzyme systems in muscle physiology and the manner in which vitamin E influences these processes, little more can be said concerning the real function of the vitamin in the animal organism.

It is too early to speculate as to whether tocopherols have a single or multiple function within the animal body, exclusive of the rôle which they may play as antioxidants. Whether the latter property accounts for their vitamin-A-sparing action in the gut wall is not yet clear.

What have seemed for many years to be irreconcilable findings now seem to fit into some more rational pattern. In fact, the structural and biochemical alterations characterizing the manifestations of vitamin E deficiency in a wide variety of animal types, largely by virtue of their striking dissimilarities, have served as important landmarks guiding investigators toward a common focus encompassing many new problems for future

investigation. It appears that these will center around the relations of tocopherols to the absorption and utilization of lipids, to products formed in the intermediary metabolism of fats (perhaps carbohydrates and proteins also), and to oxidative mechanisms essential to the structural and functional integrity of muscle and other tissues.

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The Chemistry and Physiology of Vitamin A

By I. M. HEILBRON, W. E. JONES, AND A. L. BACHARACH

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The authors regret that owing to war-time conditions it has not been possible to make this survey as exhaustive as they would have wished for the years 1940-43.

I. HISTORICAL

Although cod liver oil was in general medicinal use in Western Europe by 1840, it is only since the recognition of the existence of the deficiency diseases that progress has been made regarding the nature of the constituents responsible for its therapeutic action. It is unnecessary to discuss here

the earlier work dealing with this question, detailed accounts of which will be found in Sherman and Smith's Monograph (1) and the Medical Research Council's Survey of the Vitamins (2).

In 1913, McCollum and Davis (3) found, in agreement with earlier observations of Hopkins (4), that whereas growth ceased prematurely in rats fed on synthetic diets in which the fat was supplied as lard, it was immediately resumed on addition of either butter-fat or an ether extract of egg-yolk, and they concluded that this effect might be due to the presence of a necessary organic complex of the lipins or substances associated with the latter. Almost simultaneously Osborne and Mendel (5, 6) observed that while growth could not be maintained on a diet of "protein-free milk" it was resumed on addition of either butter or cod liver oil. Further work by McCollum and Davis (3, 7, 8, 9, 10) and by Osborne and Mendel (11) established that two distinct types of food accessory substances are essential for adequate nutrition during growth, one being soluble in fats ('fat-soluble A') and the other in water ('water-soluble B').

The next outstanding development was the demonstration by Mellanby (12) that rickets is a deficiency disease, and he succeeded in establishing that it is due to shortage of a factor which in distribution closely resembles the 'fat-soluble A' of McCollum and Davis, being present in both butter and cod liver oil. It therefore became accepted that cod liver oil possesses the following physiological properties: (a) promotion of growth and (b) the power of rapidly curing rickets, and although both these properties were originally ascribed to the same factor, evidence that the growth-promoting action was only correlated to a limited extent with the anti-rachitic activity rapidly accumulated. Thus McCollum, Simmonds, Becker, and Shipley (13) demonstrated that whereas the anti-xerophthalmic activity of cod liver oil, which had been associated with the 'fat-soluble A' some years previously, was completely destroyed by aeration for twenty hours at 100°C., its anti-rachitic activity remained unimpaired. Compare also Goldblatt and Zilva (14) and Lesné and Vagliano (15). It therefore became evident that cod liver oil contains two distinct accessories which became known as vitamins A and D. In addition to the properties ascribed above to vitamin A, Holm (16) and later Tansley (17) associated the condition of night-blindness with its deficiency (see Section IX).

As regards the chemical nature of vitamin A, McCollum and Davis (18) had meanwhile shown that the growth-promoting factor was stable to alkaline hydrolysis and could be extracted from saponified butter-fat by means of a mixture of olive oil and ether. The fact that the vitamin was concentrated in the unsaponifiable matter was subsequently confirmed by numerous investigators, and this observation has formed the basis of the majority of the attempts to isolate the vitamin, as described in Section IV.

II. COLOR REACTIONS AND SPECTROGRAPHIC ANALYSIS

As shown by Drummond and Zilva (19) considerable variations occur in the potency of cod liver oil, and a rapid method of comparison, avoiding the laborious and often inaccurate biological test, became desirable. In 1920, Rosenheim and Drummond (20) observed that although there appeared to be some association of growth-promoting powers with the presence of lipochromes, as suggested by Steenbock and Boutwell (21), they were unable to identify the vitamin with any known member of that class. They found, however, that the majority of liver oils, whether from fish, mammal, or bird, normally contain a chromogen giving a purple color with sulfuric acid and subsequent work by Drummond and Watson (22) suggested a relationship between this chromogen and vitamin A. Unfortunately, the transient nature of the color prevented its use for quantitative comparisons but Rosenheim and Drummond (23) later found that a solution of arsenic trichloride in chloroform gave an intense blue color persisting sufficiently long to allow of a colorimetric comparison with a suitable standard. The reaction was found to be extremely sensitive and there appeared to be a definite parallelism between the color intensity and the growth-promoting power. In addition the ability to give the reaction disappeared with the vitamin activity when a current of air was passed through the cod liver oil at 100°C. A further important refinement was later introduced by Carr and Price (24) who substituted antimony trichloride for the arsenic trichloride, the resulting blue color being considerably more stable and permitting of a comparison of oils and concentrates in a tintometer. Although many color reactions have been devised for identifying the vitamin, the majority are by no means specific, and in an examination of sixteen such reactions, Cocking and Price (25) concluded that the most reliable was that using antimony trichloride. This reaction gained in specificity when the blue color was described by Gillam and Morton (26) in terms of its absorption bands. These authors demonstrated that the color obtained with cod liver oil is generally characterized by maxima at 606 and 573 $m\mu$ which are respectively displaced to 617 and 583 $m\mu$ after saponification.

The first mention of a relationship between growth-promoting activity and selective absorption in the ultra-violet is due to Takahashi, Nakamiya, Kawakami, and Kitasato (27), who observed that concentrates obtained from cod liver oil exhibited selective absorption in the region of 320 $m\mu$. Subsequently Morton and Heilbron (28) found that oils which biological test proved to be rich in the vitamin exhibit a broad band in the region 300-350 $m\mu$ with a maximum near 328 $m\mu$ and that the material responsible for this band passes into the unsaponifiable fraction on alkaline hydrolysis. Furthermore, they found that the intensity of this band was in all cases approxi-

mately proportional to the intensity of the antimony trichloride blue color and that when oils were aerated or irradiated with a light source emitting rays near $320\text{ m}\mu$, the absorption band at $328\text{ m}\mu$ and the capacity to give the blue color disappeared at the same rate. The latter result supplemented the work of Peacock (29) and of Willimott and Wokes (30) dealing respectively with the destruction of the vitamin by white light and by irradiation with a mercury vapor lamp.

Extending their work to include the biological estimation, Morton and Heilbron (28) and later Drummond and Morton (31) and Morton, Heilbron and Spring (32) found that, allowing for the difficulties inherent in the biological method, there appeared to be reasonably good agreement between the growth test, the intensity of the absorption band at $328\text{ m}\mu$ and the blue color as measured photometrically. Chevallier and Chabre (33) in a similar comparison of the bio-assay and the intensity of the $328\text{ m}\mu$ band found that the latter was trustworthy providing the maximum was actually at $328\text{ m}\mu$ and not displaced by the presence of vitamin A decomposition products in badly prepared oils.

Although the above results are representative of those found for the majority of liver oils and concentrates, many anomalies have been recorded, particularly in connection with the antimony trichloride color reaction. For instance, some confusion existed before it was established that while the relation between the concentration of the oil and the intensity of the blue color is linear for the usual range of measurements, at higher concentrations this no longer holds; similarly, the intensity of the blue color is dependent on the concentration of the antimony trichloride solution. In connection with these two aspects reference should be made to the publications of Norris and Church (34, 35), Brode and Magill (36), Wokes and Willimott (37), Smith and Hazley (38) and Notevarp and Weedon (39).

While the ratio between the intensities of the two absorption bands given with antimony trichloride is reasonably constant (approximately 2:1) for potent concentrates, it varies within wide limits for liver oils and is particularly affected by the treatment of the latter, *e.g.* ageing, autoclaving, etc. It is not possible to review in this article these hitherto inexplicable and in many cases conflicting statements, and reference should be made to the publications of Mittelman (40), Steudel (41), Hawk (42) and Lovern, Creed, and Morton (43).

In addition to this natural phenomenon of variation in the ratio of the intensities of the two bands, several artificial methods of accomplishing it are known. Emmerie, van Eekelen, and Wolff (44) found that on treatment of vitamin A preparations from cod liver oil or cow's liver with either furan, methylfuran, pyrrole, indole, or skatole before addition of the

antimony trichloride, only a purple color was obtained exhibiting a single absorption band at $572\text{ m}\mu$; a similar result using 7-methylindole was subsequently reported by Morton (45). Regarding the nature of the constituent present in cod liver oil responsible for the inhibition of the $606\text{ m}\mu$ band, Heilbron, Gillam, and Morton (46) showed that it could largely be removed by mild oxidation, *e.g.* by hydrogen peroxide, benzoyl peroxide, or a trace of ozonised oxygen and since it is also removed by saponification it is possible that the agent responsible is the strongly inhibiting acid obtained from cod liver oil by Emmerie (47). A detailed review of the whole question of inhibition has recently been published by Nøtveid and Weedon (48), who also investigated the effect of the addition of small quantities of oxidizing agents to the antimony trichloride. The most satisfactory proved to be bromine (0.1 g. per litre) and with this the ratio between the 606 and $572\text{ m}\mu$ intensities becomes more constant and approaches that found for highly purified vitamin A.

In a number of oils and concentrates it has been found that in addition to the bands at 572 and $606\text{ m}\mu$, additional maxima at 645 and $693\text{ m}\mu$ manifest themselves with considerable regularity in the antimony trichloride color reaction. Apart from exceptional cases the intensities of these bands are considerably less than those for the other two and their bearing on the vitamin A problem will be discussed more fully in Section VII.

III. PRO-VITAMIN A

1. Growth-promoting Activity of Vegetable Products

Although the work described above was concerned almost exclusively with materials of marine origin, numerous reports of the association of growth-promoting activity with vegetable products are to be found in the literature. Thus Osborne and Mendel (49) demonstrated that the green parts of plants contain relatively large amounts of the 'fat-soluble A' while Coward and Drummond (50) found that green leaves exhibited a marked superiority over etiolated shoots, which were apparently inactive. On the other hand Wilson (51) stated that growth could actually be maintained if sufficient etiolated shoots were fed, a conclusion subsequently confirmed by Coward (52), Moore (53) and Heller (54), although it appeared to be definitely established from their results that the vitamin A activity increased greatly on exposure to light. From experiments with guinea pigs Hume (78) concluded that white cabbage, in contrast to the green, displayed little or no growth-promoting power, a similar result being obtained with rats by Coward and Drummond (50), who also found marked activity in green seaweeds. These results were subsequently confirmed by Collinson, Hume, Smedley-Maclean, and Smith (55) who showed

that the unsaponifiable fraction from green cabbage contains ten times as much growth factor as that from the white parts.

As the number of vegetable products shown to contain the growth-promoting substance multiplied, it became increasingly evident that some relationship existed between 'the fat-soluble A' and the presence of the plant pigments. Steenbock (56) concluded from a comparison of white and yellow corn that vitamin A was probably either a yellow plant pigment or a compound closely related to a member of this class, while Steenbock and Boutwell (21) found that whereas yellow maize will maintain normal growth in rats on a diet deficient in vitamin A, white maize is devoid of activity. Similarly, Steenbock, Sell, and Boutwell (57) showed by biological test that peas which contain the largest proportion of yellow pigment also possess the highest vitamin A activity, this correlation being emphasized by further experiments of Steenbock and Sell (58), Rosenheim and Drummond (20) and Drummond and Coward (59).

Although, as indicated above, there was a considerable weight of evidence pointing to some relation between vitamin A activity and the plant pigments, no attempt was made in the earlier work to correlate the biological activity with any specific member of the latter class, and indeed there was a certain amount of evidence indicating that although these pigments were active, they were not themselves essential for growth. Thus, while Palmer (60) was able to demonstrate that the pigments present in animal fats are carotenoids and are undoubtedly derived from the plant pigments present in the diet, the experiments of Palmer and Kempster (61) and Palmer and Kennedy (62) with fowls and rats respectively, indicated that they could be reared successfully on diets which were to all intents and purposes carotenoid-free. These experiments were of fundamental importance since they were the first to suggest that although certain members of the plant pigments might possess growth-promoting properties, the vitamin A of fish liver oils was not identical with any known carotenoid. This conclusion was supported by the observation of Stephenson (63) that the growth-promoting power of butter was not affected by complete decolorization with charcoal, a similar result being obtained later by Ahmad (64) who showed that the activity of cod liver oil remained unimpaired on complete decolorization.

2. The Biological Activity of the Carotenoids

As regards the identity of the active constituent associated with the plant pigments, Steenbock and Gross (65) demonstrated that normal growth of rats was maintained on addition of 15% of dried carrot, known to be rich in carotene, to a vitamin A free diet, while Steenbock and Bout-

well (66) found that on fractionation of the mixture of carotene and xanthophyll obtained by extraction of maize, etc., the crude carotene preparation contained the growth-promoting substance in large amount whereas the xanthophyll fraction contained little or none of it. Later Steenbock, Sell, Nelson, and Buell (67) stated in a preliminary communication that carotene which had been crystallized several times exhibited considerable vitamin A activity, although no further publication regarding this point appeared by these authors. On the other hand Rosenheim and Drummond (20) concluded that carotene is devoid of growth-promoting activity, similar negative results with carotene of various degrees of purity being recorded by Stephenson (63) and Drummond, Channon, and Coward (68). In view of this mass of contradictory evidence it is not surprising that the question of the biological activity of carotene was dismissed, the positive results which had been obtained being ascribed to the presence of the vitamin A of fish liver oils as an associated impurity. In 1928, however, the question of the possible identity of carotene with vitamin A was re-opened by von Euler, von Euler, and Hellström (69, 70) who suggested that in certain of the previous biological tests on the carotenoid pigments no provision had been made for the presence of vitamin D in the basal diet, a fact which they considered rendered the results of the earlier investigators unreliable. Their proof of the activity of carotene was subsequently confirmed by Moore (71, 72) who found that carotene, even after many crystallizations, was active in doses of 10 γ , while Collinson, Hume, Smedley-Maclean, and Smith (55) found that carotene of the highest melting-point obtainable was active in doses of 2-5 γ . In conformity with the observations of von Euler, von Euler, and Karrer (73) these authors pointed out that in all cases in which negative activity had been recorded, the biological tests had been carried out using a fat-free basal diet and they suggested that the presence of either fat or some substance in the unsaponifiable fraction of the latter was necessary for utilization of the carotene. Positive results were also obtained by Kawakami and Kimm (74) and Javillier and Emerique (75) while van Stolk, Guilbert, Pénaud, and Simmonet (76) found that carotene after eleven successive crystallizations was still active in doses of 0.002 mg. On the other hand Dulière, Morton, and Drummond (77) concluded that the activity of carotene decreased progressively as it was purified but it was later shown by Hume and Smedley-Maclean (79) that the results of these authors were vitiated by the fact that they administered the carotene in ethyl oleate in which solvent it appeared to decompose very readily. Subsequently, however, Drummond, Ahmad, and Morton (80) found carotene to be active in doses of 5 γ , so that by 1930 it was generally admitted that carotene possessed growth-promoting activity.

3. *The Relationship between Carotene and Vitamin A*

Takahashi, Nakamiya, Kawakami, and Kitasato (27) and Rosenheim and Drummond (23) had noted that carotene gave a blue color with antimony trichloride very similar to that given by materials containing vitamin A. von Euler, von Euler, and Hellström (69, 70) showed, however, by spectrophotometric comparison that these colors were not identical, the maxima being respectively at 590 and 617 $m\mu$, and they suggested that several substances might exist exhibiting the physiological activity associated with the classical vitamin A. Similarly Karrer, von Euler, and von Euler (81) suggested that growth-promoting activity may not be the characteristic of any specific substance but may be due to the presence in the molecule of the 'polyene' grouping. This view was considerably modified by von Euler, Karrer, and Rydbom (82) who found that many carotenoids which give a blue color with antimony trichloride are completely devoid of growth-promoting activity, *e.g.* lycopene, bixin, capsanthin, and fucoxanthin. While admitting the possibility that the activity of carotene might be due to the presence of an extremely active impurity, against this was the fact that the purest specimens of the hydrocarbon were also the most potent vitamin A preparations then available. In addition Moore (71) pointed out that the characteristic ultra-violet absorption band of vitamin A at 328 $m\mu$ is absent in the case of carotene and subsequently Moore (72) expressed the opinion that if the activity of carotene were due to the same compound as that present in cod liver oil, it could not fail to exhibit intense absorption in the region of 610 $m\mu$ in the Carr-Price reaction. Capper (83) also showed that the intensity of the absorption at 328 $m\mu$ in the case of carotene was insufficient to account for the activity of the latter on the basis of the presence of the classical vitamin A as impurity. Wolff, Overhoff, and van Eekelen (84) also concluded that vitamin A and carotene were not identical, and Ahmad (64) recorded that, unlike carotene, the vitamin A of cod liver oil was not readily adsorbed by charcoal.

Although von Euler and Karrer (85) considered that the excess carotene received by rats above the necessary minimum accumulated as such in the liver, von Euler, Karrer, and Rydbom (82) concluded, from the observation that carotene di-iodide also possesses growth-promoting properties, that it is not carotene itself which is the active material but some product derived from it. Since Moore (86) found that in rats fed on massive doses of carotene the latter persisted unchanged throughout the alimentary tract whereas it appeared in the form of vitamin A in the liver, it seemed likely that the actual conversion took place in that organ, and both Olcott and McCann (87) and von Euler and Klusmann (88) claim to have effected this transformation *in vitro* by means of fresh liver tissue, the

former authors suggesting that the agent responsible was an enzyme which they provisionally named carotenase. On the other hand Rea and Drummond (89) obtained negative results under these conditions, and a critical survey by Woolf and Moore (90) of the technique employed in the test on which the original claim was made indicates that further confirmation of this transformation *in vitro* is desirable.

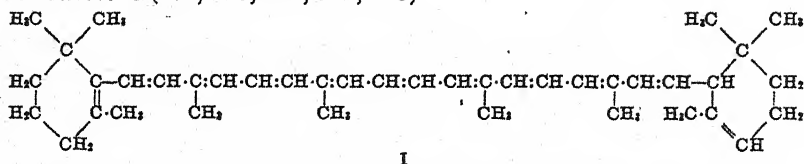
4. The Constitution of Carotene

In the above account of the biological activity of carotene, the latter had been considered as a single entity in conformity with the general belief prior to 1931. For some years previously, however, different melting-points for 'carotene' had been recorded by various authors depending on the source from which it was isolated. Thus Collinson, Hume, Smedley-Maclean, and Smith (55) found the melting-points of specimens of carotene prepared from cabbage, spinach, and carrots to be 178°, 163-164° and 164-169° respectively, while Moore (91) reported 162° and 174° for those from palm oil and carrots respectively. In 1931, Kuhn and Lederer (92, 93, 94) and Kuhn and Brockmann (95) stated that although optically inactive specimens of carotene could be isolated from winter spinach, grass, and stinging nettle, in general carotene preparations, *e.g.* from palm oil, carrots, *Sorbus aucuparia*, or *Aesculus hippocastanum* are dextro-rotary even after repeated crystallization. They demonstrated, however, that such optically active preparations could be separated by chromatographic adsorption and fractional precipitation with iodine into two isomers which they designated α -carotene ($[\alpha]_{\text{Cd}}^{20} + 380^\circ$ in benzene) and β -carotene (optically inactive). In addition they found that decomposition of β -carotene tetra-iodide with either mercury or sodium thiosulfate gave a third isomer which they named isocarotene. Confirmation of the existence of the α - and β -carotenes was supplied by Karrer, von Euler, and Hellström (96) and van Stolk, Guilbert, and Pénau (97), while Karrer and Walker (98) subsequently showed that a single adsorption on either calcium hydroxide or lime is sufficient for complete separation of the two isomers, the β -form being the more strongly adsorbed. Subsequently, Kuhn and Brockmann (99, 100) discovered a third naturally-occurring isomer, γ -carotene, which is normally present to the extent of approximately 0.1% in carotene as obtained from various sources, although Winterstein (101) has shown that it constitutes some 50% of the total carotenoids present in the fruit peel of *Gonocaryum pyriforme*.

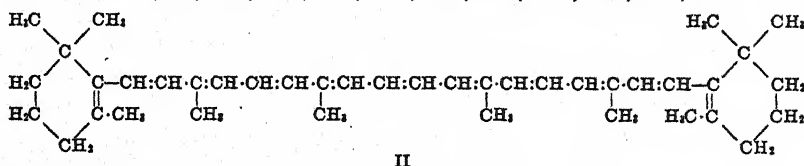
It is not proposed to discuss in detail the chemistry of the isomeric carotenes except in so far as it bears on the vitamin A problem and it is considered sufficient merely to summarize the salient points. For more detailed accounts the reader is referred to the reviews by Bogert (102),

von Euler (103, 104), Kuhn (108) and Mackinney (105), the Chemical Society's Annual Reports on the Progress of Chemistry (106) and Zechmeister's monograph (107). The formulae of, and the principal references relating to, these compounds are as follow:

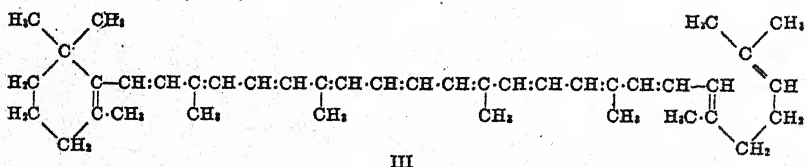
α -Carotène (109, 110, 111, 112, 113)



β -Carotène (109, 114, 115, 116, 117, 118, 119, 120, 121, 122)

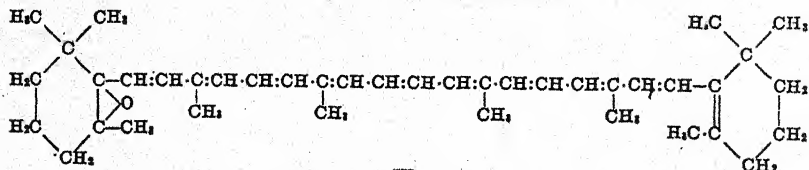


γ -Carotene (99, 100)

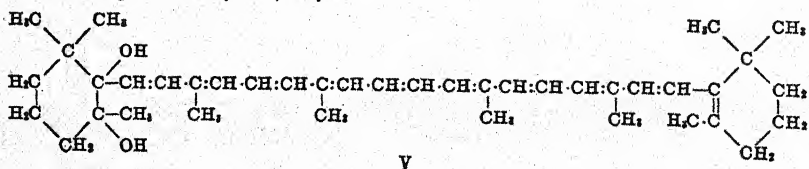


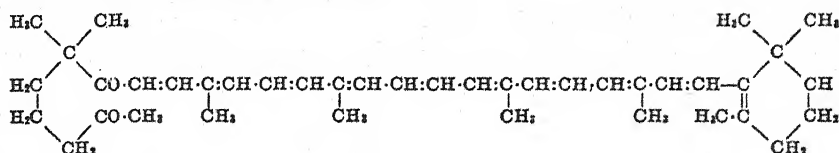
All three isomers possess growth-promoting properties, and Kuhn, Brockmann, Scheunert, and Schieblich (123) established that β -carotene is twice as active as the α - and γ -isomers. Other compounds possessing vitamin A activity include:

(a) β -Carotene oxide (IV) obtained by von Euler, Karrer, and Walker (124) by the action of perbenzoic acid on β -carotene.



(b) β -Oxycarotène, V (125)



(c) Semi- β -carotenone, VI (120)

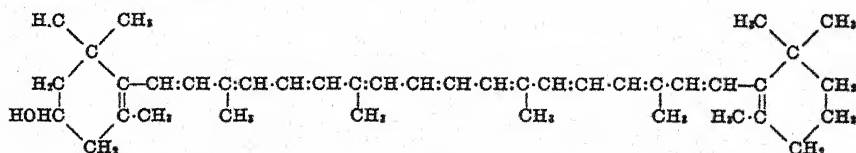
VI

TABLE I

Properties of Carotenoids

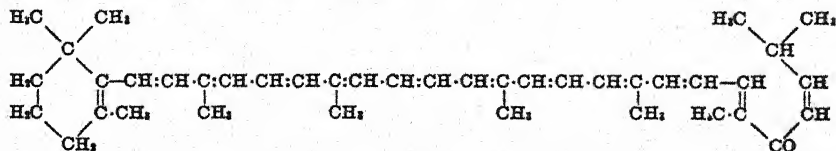
	Δ	Unsub. β -ionone Rings	λ_{\max} in CS_2			Vitamin A Activity
α -Carotene.....	11	1	511	478		Active
β -Carotene.....	11	2	521	485.5		Active
γ -Carotene.....	12	1	533.5	496	463	Active
β -Oxycarotene.....	10	1	508	475	446	Active
β -Carotenone.....	9	0	538	499	466	Inactive
Semi- β -carotenone.....	10	1	538	499		Active
β -Carotene oxide.....	10	1	486	456	427	Active
α -Oxycarotene.....	10	0	502	471	440	Inactive
Kryptoxanthin.....	11	1	519	483	452	Active
Lutein.....	11	0	511	479	446	Inactive
Zeaxanthin.....	11	0	515	485		Inactive
Lycopene.....	13	0	548	507.5	477	Inactive

(d) Kryptoxanthin, for which Kuhn and Grundmann (126) have suggested formula VII.



VII

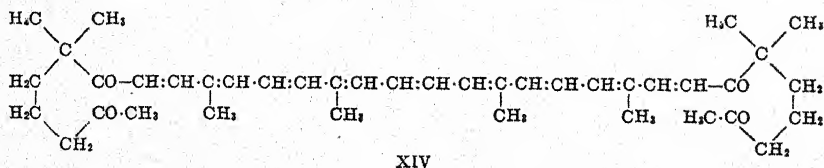
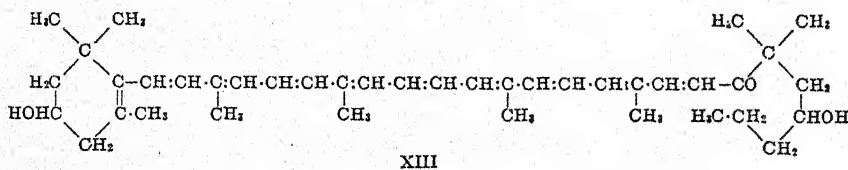
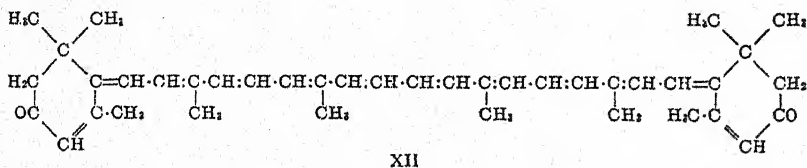
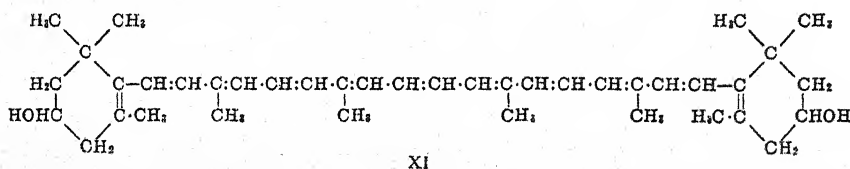
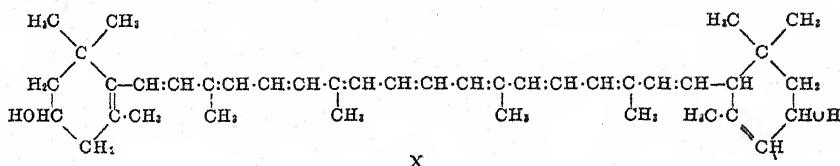
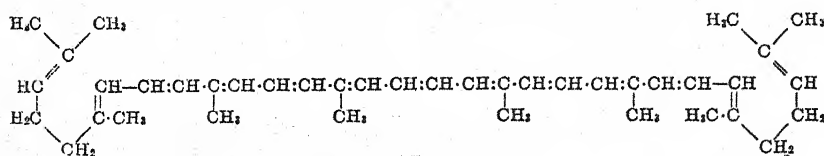
(e) Myxoxanthin, isolated by Heilbron and Lythgoe (127) from *Oscillatoria rubescens*, and possessing structure VIII.



VIII

On the other hand lycopene (IX), lutein (X), zeaxanthin (XI), rhodoxanthin (XII), capsanthin (XIII), and β -carotenone (XIV) are all biologically inactive, and from a comparison of the properties of the common carotenoids listed in Table I it will be seen that although the biological activity

is not entirely dependent on the number of double bonds, the presence of a β -ionone ring would appear essential, a possibility first suggested by Karrer, Schöpp, and Morf (128).



Details of the absorption spectra of the carotenes and vitamins A have been summarized by Loofbourow (378).

Although the dihydrocarotenes and carotene iodides display growth-

promoting activity, it is believed that this is due to the regeneration of the parent α - or β -carotene in the body (Kuhn, 108).

In addition to the above compounds, Karrer and his colleagues have obtained several degradation products of β -carotene containing fewer than forty carbon atoms but still showing biological activity. These will be discussed in greater detail in Section VII.

Although several investigators, including Willimott and Moore (129) and von Euler, Karrer, and Zubrys (130), have shown that xanthophyll (lutein) cannot replace vitamin A in the diet of rats it has been claimed by von Euler and Rydbom (131), though not yet confirmed by any other authors, that carotene may be replaced by xanthophyll as a source of vitamin A in chickens but not in rats owing to incomplete absorption in the latter animals.

IV. ISOLATION OF PURE VITAMIN A

As already mentioned in Section I, McCollum and Davies (18) established that vitamin A could be concentrated in the unsaponifiable fraction of butter-fat, and Takahashi (132), Takahashi, and Kawakami (133) and Takahashi, Nakamiya, Kawakami, and Kitasato (27) applied this discovery to the case of cod liver oil. They found the small amount of unsaponifiable matter obtained on alkaline hydrolysis to be highly potent, and from it by fractional distillation they claimed to have isolated the active principle, which they named biosterin. This material proved to be biologically active in daily doses of 80 γ , but later work by Drummond, Channon, and Coward (68) and Nakamiya and Kawakami (134) established beyond doubt that it was in fact grossly impure. In an examination of a Japanese shark liver oil with a vitamin A content (as estimated by the Carr-Price test) corresponding to a good cod liver oil, Drummond and Baker (135) definitely accounted for 90-95% of the unsaponifiable fraction as well-known inactive substances such as selachyl, batyl, chimyl, and oleyl alcohols, together with a small quantity of cholesterol and a hydrocarbon resembling squalene. As a result of this work these authors concluded that "the active substance is present in cod liver oil concentrates in amount so minute that direct attempts at its isolation by ordinary chemical means are of little use."

In a search for more potent sources of the vitamin a large number of fish liver oils were investigated by various workers. Poulson (136) found that halibut liver oil was very rich in vitamin A, while Schmidt-Nielssen and Schmidt-Nielssen (137) showed that the antimony trichloride blue values of halibut (*Hippoglossus hippoglossus*), mackerel (*Scomber scombrus*), salmon (*Salmo salar*), red perch (*Sebastes marinus*) and cod liver oils were in the ratio 700:570:250:70:6 and these authors made the further

interesting observation that those liver oils which could only be isolated by solvent extraction invariably gave a higher blue value than those obtained by steaming. Similar results regarding the relative richness of halibut liver oil were subsequently obtained by von Euler and Karrer (138) and Lovern (139), the latter author stating that although wide variations in potency were common, the liver oil of *Hippoglossus vulgaris* is generally 50-100 times richer in vitamin A than a good cod liver oil, while Pett, Lipkind, and LePage (140) have stated that ling cod liver oil is even more potent than halibut liver oil. The values shown in Table II, recently quoted by Jewell, Mead, and Phipps (141) exemplify the wide variation encountered in the average vitamin A content of various fish liver oils.

More recently the researches of Lovern, Edisbury, and Morton (142), Edisbury, Lovern, and Morton (143) and Edisbury, Morton, Simpkins, and Lovern (144) have established that the oils from the visceral organs of various fish are frequently richer sources of the vitamin than the liver oils,

TABLE II
Vitamin A Content of Fish Liver Oils

Name	Zoological Name	Approx. vitamin A content, %
Cod	<i>Gadus morrhuas</i>	0.01
Conger eel	<i>Conger vulgaris</i>	0.2
School shark	<i>Galcorhinus australis</i>	0.7
Halibut	<i>Hippoglossus hippoglossus</i>	1.7
Tunney	<i>Thunnus vulgaris</i>	4.7

while Lovern, Mead, and Morton (145) and Lovern and Morton (146) have shown that large deposits of vitamin A, almost exclusively in the form of esters, occur in the intestines of the halibut where it is strictly localized in the mycosa and in particular in the *tunica propria*.

Contrary to the experiences of Drummond and Baker (135) with cod liver oil, von Euler and Karrer (138) and Karrer, Klussmann, and von Euler (147), starting from a rich halibut liver oil, found that saponification, followed by removal of the major portion of the sterols by freezing in methyl alcohol, gave a concentrate which they estimated to contain at least 50% vitamin A. Further purification has been effected by various investigators employing both chromatography and molecular distillation.

(a) *Chromatographic Adsorption.* While the above preliminary work was being carried out, the investigation of the carotenoid pigments had proceeded with increasing momentum, and in view of the apparent relationship between β -carotene and vitamin A, it seemed logical to apply the experience gained in the carotenoid field to the case of vitamin A. The

value of chromatography in its purification was first demonstrated in a preliminary note by von Euler and Karrer (138) who showed that the Carr-Price value of 6,000 cod liver oil units (= 60,000 blue units) obtained for a concentrate prepared from a halibut liver oil, was raised to 10,000 C.L.O. units after a single adsorption, and in a subsequent publication Karrer, Morf, and Schöpp (148) described in detail the concentration of the vitamin by this method starting from the liver oil of *Hippoglossus hippoglossus*. The total unsaponifiable fraction was obtained as a semi-solid mass from which practically the whole of the sterol was removed by cooling in methyl alcohol first at -15° and then at -60° , the mother-liquor yielding a clear yellow oil with a Carr-Price value of 8,000 C.L.O. units. For the subsequent chromatographic purification a solution of this material in petroleum ether was allowed to pass slowly down a tube filled with Merck's Fasertonerde and finally washed down the column with the same solvent. The vitamin was mainly adsorbed on the middle third of the column, and elution with a mixture of petroleum ether and methyl alcohol gave a concentrate with a value of 9,100 C.L.O. units, rising to 10,500 on a second adsorption. No further purification could be obtained by repeated adsorption, and the concentrate so obtained was an extremely viscous golden-yellow oil which was readily soluble in the common organic solvents and which readily oxidized in air. Biologically it was found to be active in doses of 0.5 γ (see correction of Karrer, Morf, and Schöpp, 149) thus being approximately ten times more potent than the purest specimens of carotene available at that time. The latter authors also examined the concentrate prepared by the above process from the liver oil of *Scombrex saurus* (a variety of mackerel) and found that the product was indistinguishable from that obtained above, from which they concluded that the concentrates so obtained might be regarded as approximately homogeneous. It was subsequently suggested by Karrer that vitamin A should be given the name Axerophthol.

Holmes, Cassidy, Manly, and Hartzler (150) later published details regarding adsorption of the vitamin on a special activated charcoal, and they claimed that by this method, followed by adsorption on magnesium oxide, they were able to obtain concentrates with blue values as high as 140,000. However, it must be pointed out that the use of the colorimetric method of assay detracted somewhat from the value of this work, owing to the relatively large increase which can occur in the blue value on removal of small quantities of anti-oxidants. The necessity of storing vitamin A concentrates in a solvent was incidentally shown by these authors, who found that their richest material, even on keeping in an inert atmosphere in solid carbon dioxide, rapidly became crystalline and then gave only a feeble color with antimony trichloride.

(b) *High Vacuum Distillation.* As already mentioned, attempts to distil the vitamin had been made by Drummond and Baker (135) who found that even at pressures as low as 0.01 mm. extensive decomposition of the vitamin occurred. Similar results were obtained by Heilbron, Heslop, Morton, Webster, Rea, and Drummond (151) at pressures below 10^{-4} mm., but in collaboration with Carr and Jewell of British Drug Houses Ltd., these authors found that the vitamin could be successfully distilled in a specially constructed molecular still, in which rapid fractionation could be effected at pressures below 10^{-3} mm. Under these conditions no decomposition occurred, and a fraction, b.p. 137–138°, having considerably enhanced potency, contained the major portion of the vitamin.

In addition to halibut liver oil, Heilbron and his colleagues (151) also investigated sturgeon liver oil and a mammalian liver fat, and Table III

TABLE III
Highest Values Obtained for Concentrates from Various Sources

Concentrate	$E_{1\text{ cm}}^{1\%}$			
	328 m μ	617 m μ	580 m μ	693 m μ
Halibut (distillate).....	1370	4650	2550	450
Another halibut (adsorbed distillate).....	1350	4350	2350	900
Sturgeon (best distillate).....	1330	4200	2130	1020
Mammalian non-sap. (adsorbed).....	1300	3810	1995	No band
Mammalian non-sap. (distillate).....	1250	3620	2350	No band
Prof. Karrer's halibut preparations: (a).....	1350	4130	2250	364
(b).....	1250	3250	1600	300

records the highest values obtained for the concentrates from the various sources, together with those for two samples prepared by the chromatographic method kindly supplied by Professor Karrer for direct comparison. From these it will be seen that, as regards the ultra-violet absorption, the products from the various sources and employing the two different processes were indistinguishable. One disturbing factor, however, was that certain preparations contained a chromogen giving rise to an absorption band at 693 m μ with antimony trichloride. The nature of this chromogen will be discussed more fully in Section VII.

Subsequently, Carr and Jewell (152) obtained a concentrate by distillation in a vacuum of the order of 10^{-5} mm. with a value of $E_{1\text{ cm}}^{1\%} = 1600$ and a blue value of 78,000; 0.6 γ of this preparation cured xerophthalmia and gave a slightly better growth response than 1 γ of International Standard carotene.

Recently the molecular distillation of fish liver and visceral oils has as-

sumed a considerable potential importance commercially, and a review of this subject has been published by Jewell, Mead, and Phipps (141). For descriptions of the various designs of molecular still and their application, reference should be made to recent articles by Burch and van Dijk (153), Fawcett (154), Burrows (155), Hickman (156), and Hickman and Hecker (157).

(c) *Crystalline Vitamin A and Derivatives.* Vitamin A was first isolated in crystalline form by Holmes and Corbet (158) by prolonged cooling to -60° of a slightly aqueous methyl alcoholic solution of rich concentrates, the latter being obtained from the liver oils of *Stereolepis ishinagi* and Atlantic mackerel. The vitamin so obtained was in the form of pale yellow rosettes of needles, m.p. $7.5-8.0^{\circ}$, with an extinction coefficient $E_{1\text{ cm}}^{1\%} 328\text{ m}\mu = 2,100$, and biological tests proved its activity to be of the order of 3×10^6 International units per g.

A similar material was subsequently isolated by Mead (159) from a distilled halibut liver oil concentrate, but more recently Baxter and Robeson (160) have established that the crystals obtained by this means contain approximately 10% of methyl alcohol of crystallization. Starting from shark liver, ling cod liver, Californian jewfish liver, and halibut viscera oils having extinction coefficients $E_{1\text{ cm}}^{1\%} 328\text{ m}\mu = 100, 129, 317$ and 72 respectively, they obtained the vitamin in the form of prisms m.p. $63-4^{\circ}$, by distillation of the oil, saponification and crystallization at low temperature from ethyl formate. They also showed that this material and the form m.p. $7-8^{\circ}$ are interconvertible by crystallization from the appropriate solvent and that both give the same derivatives. There seems little doubt that the product obtained by crystallization from ethyl formate is the pure vitamin despite the fact that the extinction coefficient, $E_{1\text{ cm}}^{1\%} 328\text{ m}\mu = 1750$, is considerably lower than the value quoted by Holmes and Corbet (158). It is in better agreement than the latter, however, with the figure of 1880 predicted by Morton (161).

It has already been mentioned that early attempts by Karrer, Morf, and Schöpp (149) and Heilbron, Heslop, Morton, Webster, Rea, and Drummond (151) to prepare solid derivatives such as the acetate, benzoate, and *p*-nitrobenzoate were unsuccessful. In 1935, however, Hamano (163, 164) succeeded in preparing, from concentrates obtained from the liver oils of *Theragra chalcogramma*, *Stereolepis ishinagi*, *Sebastes flammus* and *Thynnus alalunga*, a β -naphthoate of vitamin A, m.p. 76° and an anthraquinone-2-carboxylate, m.p. 124° , the same derivatives later being prepared by Mead (165). Recently, however, Baxter and Robeson

(166), starting from crystalline vitamin A have prepared four solid esters having the following properties:

(a) Acetate. Pale yellow prisms from methyl alcohol. M.p. 57-58°, $E_{1\text{ cm}}^{1\%} 328\text{ m}\mu = 1510$.

(b) Palmitate. Yellow prisms from propylene oxide. M.p. 27-28°, $E_{1\text{ cm}}^{1\%} 328\text{ m}\mu = 940$.

(c) Succinate. Yellow prisms from ethyl formate. M.p. 76-77°, $E_{1\text{ cm}}^{1\%} 328\text{ m}\mu = 1240$.

(d) β -Naphthoate. Yellow prisms from ethyl alcohol. M.p. 74-75°, $E_{1\text{ cm}}^{1\%} 328\text{ m}\mu = 1090$.

A further type of solid derivative was prepared by Kawakami (167) who acetylated a concentrate from the liver oil of *Theragra chalcogramma* and condensed the product with maleic anhydride in benzene. A crystalline solid, m.p. 261-262°, was obtained analyzing for the di-maleic adduct of acetylated vitamin A, and a similar compound, m.p. 263-264°, prepared in the same way from the benzoylated concentrate. This work was extended by Hamano (162, 164) to the liver oils themselves, those from *Stereolepis ishinagi*, *Theragra chalcogramma* and *Sebastodes matsubarae* giving a compound m.p. 220°, analyzing as the di-maleic adduct of vitamin A palmitate. This was later confirmed by Tischer (168) who obtained the same compound by molecular distillation of cod liver oil followed by condensation with maleic anhydride. This investigator, on the basis of this work, estimated that vitamin A palmitate constituted some 3% of the total esters present in the liver oil.

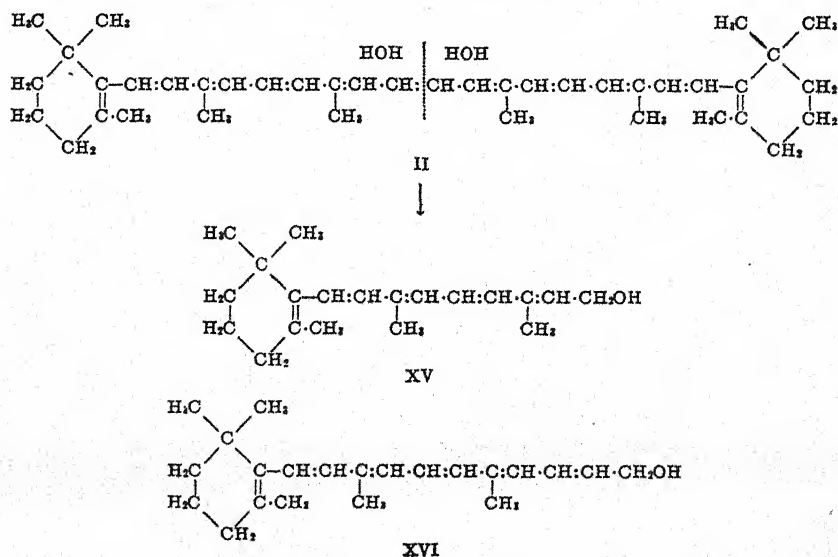
V. CONSTITUTION OF VITAMIN A

1. Degradation

Although, as described in Section III, it had been shown that carotene and vitamin A were not identical, there seemed reasonable grounds for concluding that the two were in some way related. That, however, a simple chemical relationship existed between them seemed to be improbable, since Bruins, Overhoff, and Wolff (169), by direct comparison of the diffusion constants of carotene and a rich vitamin A concentrate prepared from sheep liver fat, calculated the molecular weight of the vitamin to be 333 as against 536 for carotene. Values of 300-320 were also obtained by Karrer, Morf, and Schöpp (149) using the Rast method, while Heilbron, Heslop, Morton, Webster, Rea, and Drummond (151) found that cryoscopic determinations gave a mean value of 327 and the Smith and Young (170) modification of the Rast micro-method 312.

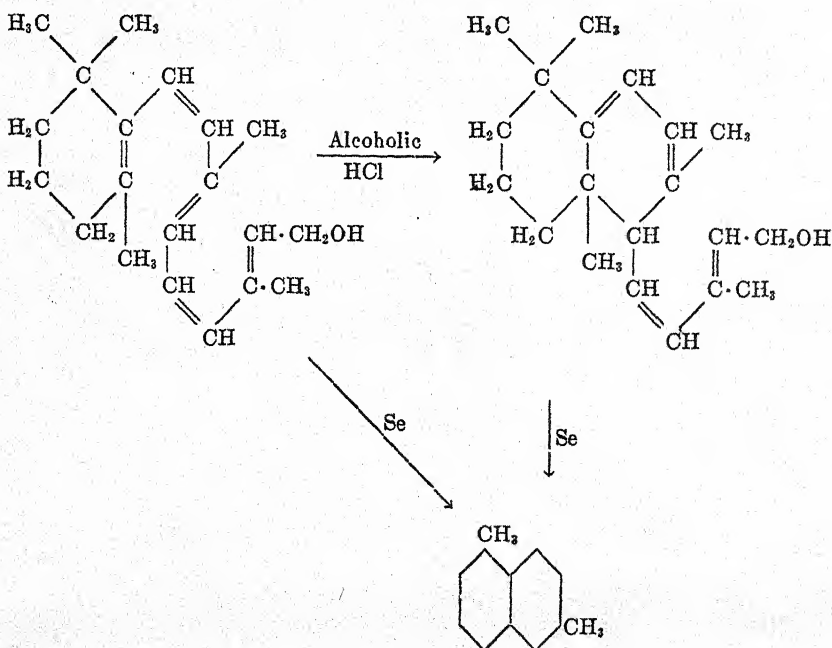
It has already been mentioned that Drummond, Channon, and Coward (68) believed vitamin A to be an alcohol, and this was confirmed by Karrer, Morf, and Schöpp (149) who showed that the rich concentrate prepared from the liver oil of *Scombrox saurus* formed an acetate and a *p*-nitrobenzoate, both of which, however, were viscous oils. Analytical data for these two derivatives, and for the alcohol regenerated from the acetate, indicated that the molecular formula of the vitamin was most probably $C_{20}H_{30}O$ or $C_{22}H_{32}O$. Similar results were obtained later by Heilbron Heslop, Morton, Webster, Rea, and Drummond (151) for the distilled concentrate, and it will be noted that the respective molecular weights of 286 and 312 for these formulae are in fair agreement with the values found experimentally. The presence of a β -ionone ring in the vitamin was established by Karrer, Morf, and Schöpp (148) who found that ozonolysis gave geronic acid in amount indicating the presence of one β -ionone ring, and these authors (148) also found that quantitative oxidation with potassium permanganate and with chromic acid yielded acetic acid in amounts corresponding to 9.7 and 16.3% of C-methyl respectively. They also showed that the concentrate was optically inactive and on hydrogenation absorbed 4.9 mols. of hydrogen calculated on a molecular weight of 286 (or 5.4 mols., for a molecular weight of 312), the analysis of the hydrogenated product agreeing with either of the formulae $C_{20}H_{40}O$ or $C_{22}H_{44}O$.

On the basis of the above evidence Karrer, Morf, and Schöpp (149) suggested the two alternative formulae, XV and XVI, as possible structures for the vitamin, the former being regarded as the more probable since it represents exactly half the β -carotene molecule.



Considerable support for the structure XV was supplied by a consideration of the absorption spectrum of the vitamin. By comparison of octatrienol, decatetraenol, dihydrocrocetin, and dihydrobixin methyl ester containing respectively three, four, six and eight conjugated ethylenic linkages, von Euler, Karrer, Klusmann, and Morf (171) showed that the position of the absorption band of the vitamin lies between those of decatetraenol and dihydrocrocetin, thus pointing to its containing five and not six double bonds.

Additional indirect proof of the correctness of the above type of formulation was supplied by Heilbron, Morton, and Webster (172). It had already been shown by Edisbury, Gillam, Heilbron, and Morton (173) that vitamin A is extremely sensitive towards mineral acids, the product obtained exhibiting highly characteristic absorption bands, and it was believed that the mechanism of the change might involve a partial cyclization. The former authors found that dehydrogenation of this product with selenium gave a good yield of 1,6-dimethyl-naphthalene, which was also obtained by a similar treatment of the vitamin itself. The isolation of 1,6-dimethyl-naphthalene definitely establishes the terpenoid nature of the vitamin and the presence in the richest concentrates of a material possessing a constitution which, as far as the fourteenth carbon atom, must be identical with the structures XV and XVI.

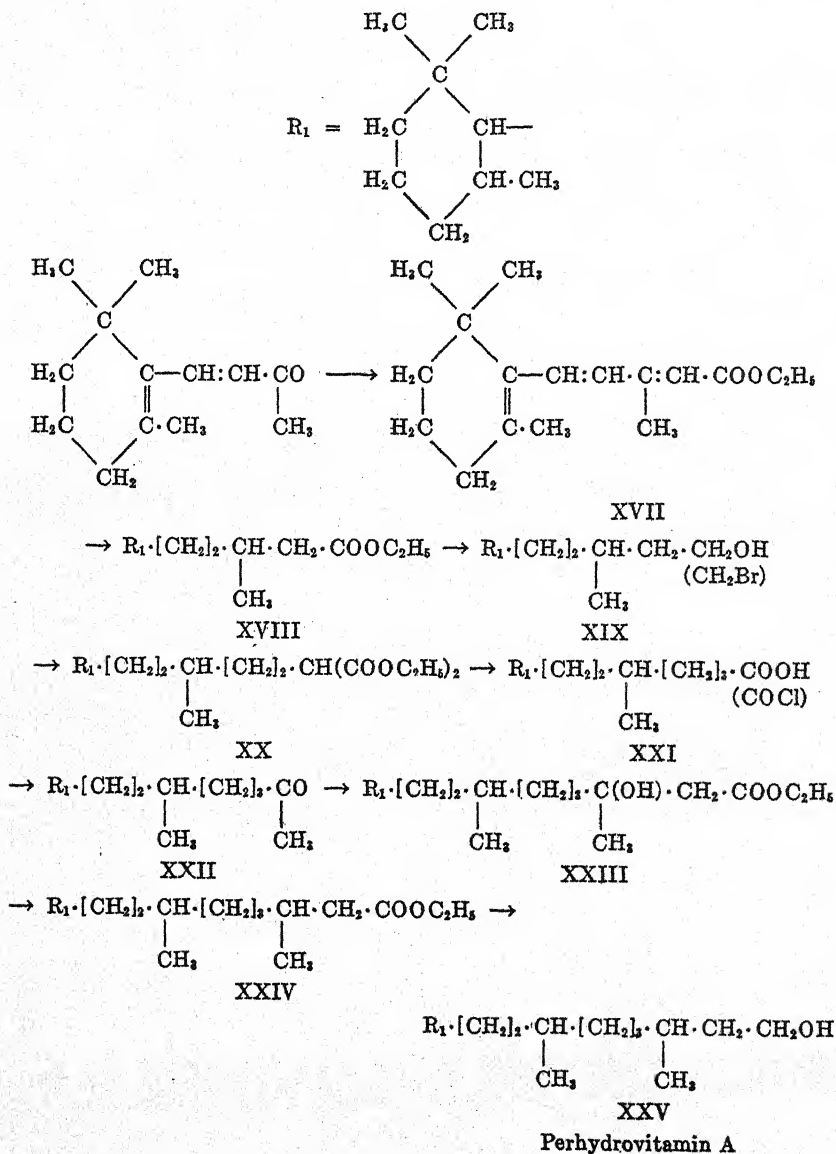


It has been mentioned above that an absorption maximum at $693\text{ m}\mu$ is generally present, at varying intensities, in the antimony trichloride blue color, but it was observed by Heilbron, *et al.* (151) that the ratio between the intensities of absorption at 580 and $617\text{ m}\mu$ was relatively constant even in the most potent preparations and it seemed likely that both were characteristic of the vitamin. This was supported to some extent by the generally accepted belief that β -carotene also exhibited bands at 542 and $590\text{ m}\mu$ with antimony trichloride, but it was later shown by Karrer, Walker, Schöpp, and Morf (174) that α - and β -carotenes which had been carefully purified chromatographically exhibited only one band each, at 542 and $590\text{ m}\mu$ respectively. Extending this work to vitamin A these authors and von Euler, Karrer, and Zubrys (130) investigated the adsorption of their richest concentrates on calcium oxide and hydroxide. The main, or β -fraction, which was not adsorbed, exhibited the characteristic maximum at $328\text{ m}\mu$ and a single band at $622\text{ m}\mu$ in the antimony trichloride color. In addition its analysis agreed exactly with the formula $\text{C}_{20}\text{H}_{30}\text{O}$, it gave geronic acid on ozonolysis and was active in doses of $0.3\text{ }\gamma$ daily. The remainder, or α -fraction, comprising only a few per cent of the total, was adsorbed on the column and was characterized by an absorption band at $270\text{ m}\mu$. This material, to which the name hepaxanthin was given, showed only a single maximum at $580\text{ m}\mu$ in the antimony trichloride color but a second band at $620\text{ m}\mu$ rapidly developed on standing, probably due to isomerization. Its growth-promoting power was much less than that of the main portion, and it was uncertain whether it was active *per se* or contained some of the β -fraction. The above conclusions were in accord with the earlier results of van Eekelen, Emmerie, Julius, and Wolff (175) who isolated, from a liver oil, a fraction showing no growth-promoting properties and agreeing in absorption spectrum with hepaxanthin; these investigators concluded that only the $620\text{ m}\mu$ band is characteristic of vitamin A. Castle, Gillam, Heilbron, and Thompson (176), however, were unable to effect the separation of these two chromogens by the above method, and it would appear that independent confirmation of these results is desirable.

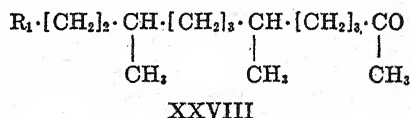
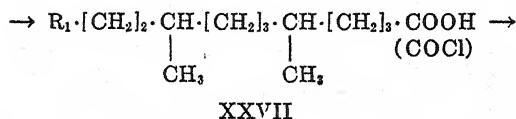
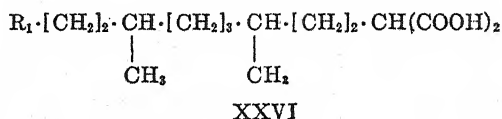
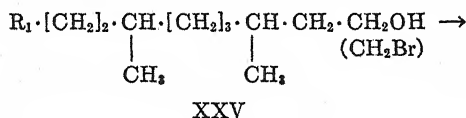
2. Synthesis of Perhydrovitamin A

That vitamin A definitely possesses the C_{20} carbon skeleton of structure XV was finally established by Karrer and Morf (177) who synthesized the corresponding saturated alcohol and compared it with the hydrogenated natural product. Karrer, Salomon, Morf, and Walker (178) had previously shown that condensation of β -ionone with ethyl bromoacetate by the Reformatsky reaction readily gave ethyl β -ionylideneacetate, XVII, which Karrer and Morf (177) hydrogenated to XVIII. Bouveault-Blanc

reduction of this ester gave the alcohol XIX and condensation of the corresponding bromide with diethyl malonate yielded the dicarboxylic ester XX, which was readily converted by the standard reactions into the C_{17} monocarboxylic acid XXI. Treatment of the acid chloride with zinc methyl iodide gave the C_{18} ketone XXII, and by a repetition of the above series of reactions this was finally converted into the C_{20} perhydroalcohol XXV.



Both the synthetic alcohol and the product obtained by hydrogenation of a rich concentrate in the presence of platinum oxide were viscous oils which failed to yield solid derivatives, so that although the agreement between the physical data for the two compounds was extremely close, conclusive proof of identity was lacking. In order to overcome this difficulty both alcohols were converted into the C_{23} ketones as indicated below and comparison of the semicarbazones, m.p. 67° , of the two products established their identity, from which it followed that the vitamin must necessarily possess the carbon skeleton of formula XV.



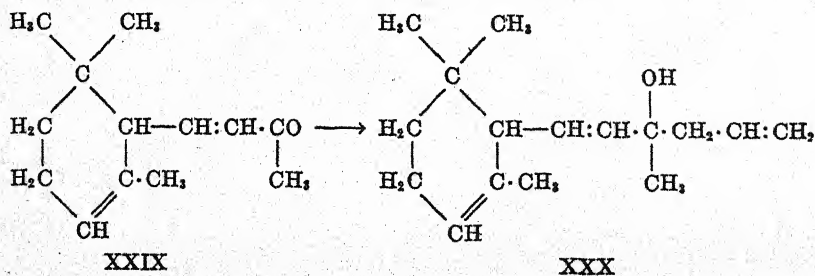
3. Possible Isomers of Vitamin A

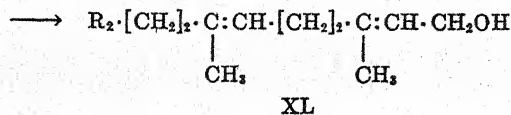
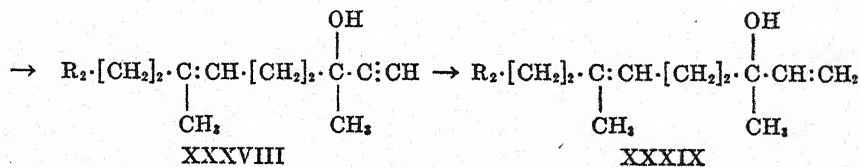
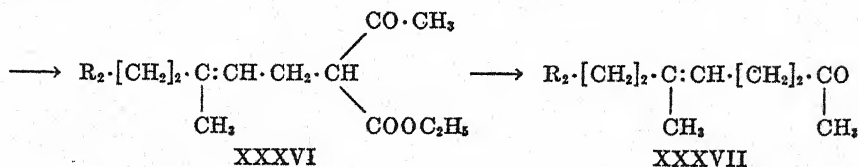
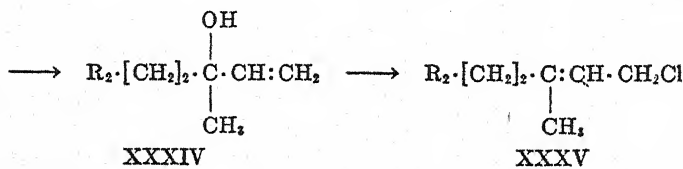
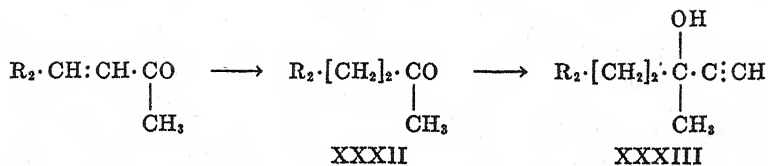
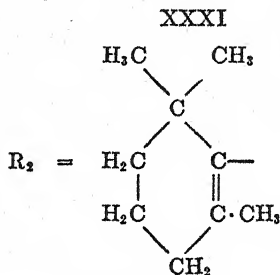
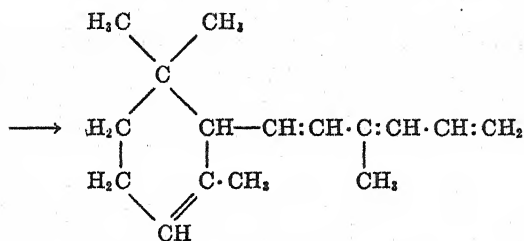
On the basis of the established formulae for vitamin A and α - and β -carotenes, it would be expected that the latter compounds would give rise respectively to one and two molecules of vitamin A in the body and this contention is supported by the observations of Brockmann and Tecklenburg (179) who fed daily doses of 1 mg. of the two carotenes to vitamin A deficient rats and then examined the liver oils. Calculated on the relative protective doses of β -carotene and vitamin A the yield of the latter was expected to be approximately 20% but in actual fact it was found to be only about 8%. On the other hand the yield from α -carotene was less than half that from the β -isomer and no trace of the isomer of vitamin A containing the α -ionone ring could be detected. Recently Mead (165) and Coward and Underhill (180) have shown that the β -naphthoate and anthraquinone-2-carboxylate of the vitamin have potencies of 2.232×10^6 and 1.77×10^6 international units per g. respectively. From these values it is calculated that pure vitamin A has a potency of

approximately 3.32×10^6 I.U. per g. which is in good agreement with the figure of 3×10^6 found for crystalline vitamin A by Holmes and Corbet (158). This value is almost double the international standard of β -carotene (1.667×10^6 I.U. per g.), from which these authors conclude that one molecule of β -carotene is converted into only one molecule of vitamin A in the body. That isomers of vitamin A do occur, however, would appear to be suggested by several observations. Thus Nakamiya (181) hydrogenated a concentrate obtained from the liver oil of *Stereolepis ishinagi* and converted it into the C_{23} ketone by the series of reactions already described. The product was characterized by a semicarbazone, m.p. 35° , in contrast to the m.p. of 67° for the derivative obtained by Karrer and Morf (177), indicating isomerism between the perhydro-alcohols prepared by hydrogenation of the vitamin obtained from two different sources. In addition Kawakami (167) found that whereas the acetylated concentrate from *Theragra chalcogramma* gave a maleic anhydride adduct m.p. $261-262^\circ$, the corresponding product from *Stereolepis ishinagi* was an isomer m.p. $221-222^\circ$, while Hamano (164) found that the mother liquors from the yellow anthraquinone-2-carboxylate, m.p. 124° , of the vitamin contained a red isomer, m.p. 118° , having the same absorption spectrum.

VI. SYNTHETIC EXPERIMENTS

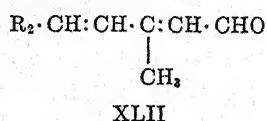
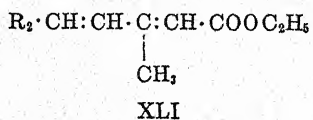
The proof of the presence of the β -ionone ring in the vitamin naturally led to the preparation of a number of compounds containing this grouping as a preliminary to the synthesis of the vitamin itself. The most convenient starting-material in this work was obviously β -ionone but its use has been complicated by the fact that it behaves anomalously in many reactions. Karrer, Salomon, Morf, and Walker (178) found that whereas α -ionone XXIX condenses with allyl bromide by the Grignard reaction to give the carbinol XXX, readily dehydrated to the hydrocarbon XXXI by means of phenylisocyanate, the corresponding reaction with β -ionone did not proceed as might be expected, apparently owing to the addition of the Grignard compound to the unsaturated system.





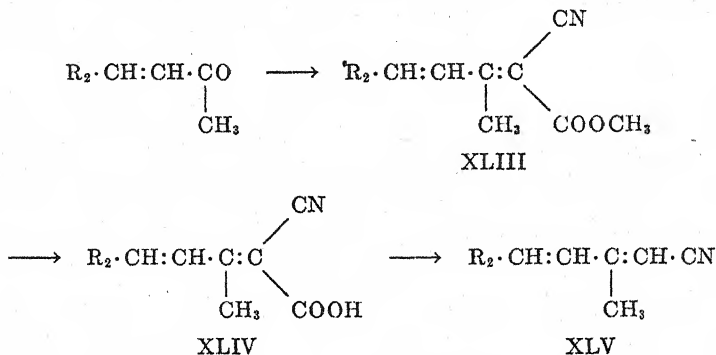
In 1934, however, Ruzicka and Fischer (182) synthesized a tetrahydro-vitamin A by the following series of reactions. β -ionone was partially hydrogenated in alcohol with nickel to dihydro- β -ionone, XXXII, and the latter condensed with acetylene in the presence of sodamide giving an excellent yield of the acetylene carbinol, XXXIII. Partial hydrogenation with nickel gave the corresponding ethylenic carbinol, XXXIV, which on treatment with phosphorus pentachloride in petroleum ether yielded the primary chloride XXXV by allylic re-arrangement, and condensation of the latter with ethyl sodio-acetoacetate gave the keto-ester XXXVI, which was hydrolyzed by aqueous alcoholic baryta to the ketone XXXVII. The acetylene carbinol, XXXVIII, obtained from the latter as for the lower homologue was hydrogenated to the ethylene carbinol XXXIX, which was then re-arranged directly with acetic anhydride to the tetrahydro-vitamin A, XL. As was expected this compound was devoid of growth-promoting activity despite the presence of the β -ionone ring, thus confirming the belief that the presence of the conjugated system of double bonds is also an essential factor.

In 1935, Davies, Heilbron, Jones, and Lowe (183) commenced an investigation into the possibility of preparing the triethenoid aldehyde XLII (β -ionylideneacetaldehyde), which they regarded as the key intermediate in the synthesis of the vitamin. Since it had been shown (178) that β -ionone condenses very readily with ethyl bromoacetate by the Reformatsky reaction giving the C_{17} ester, XLI, they investigated the distillation of the barium salt of the corresponding acid with barium formate under reduced pressure. These authors had already shown that under similar conditions citrylideneacetic acid gave the corresponding aldehyde, while Tiemann (184) had demonstrated the possibility of preparing citral from geranic acid by this method, but although an excellent yield of an aldehyde was obtained, the analyses of several derivatives of which proved it to possess the formula $C_{15}H_{22}O$, its absorption spectrum indicated only an inflexion at 280–290 $m\mu$ instead of the anticipated band in the region of 320 $m\mu$.



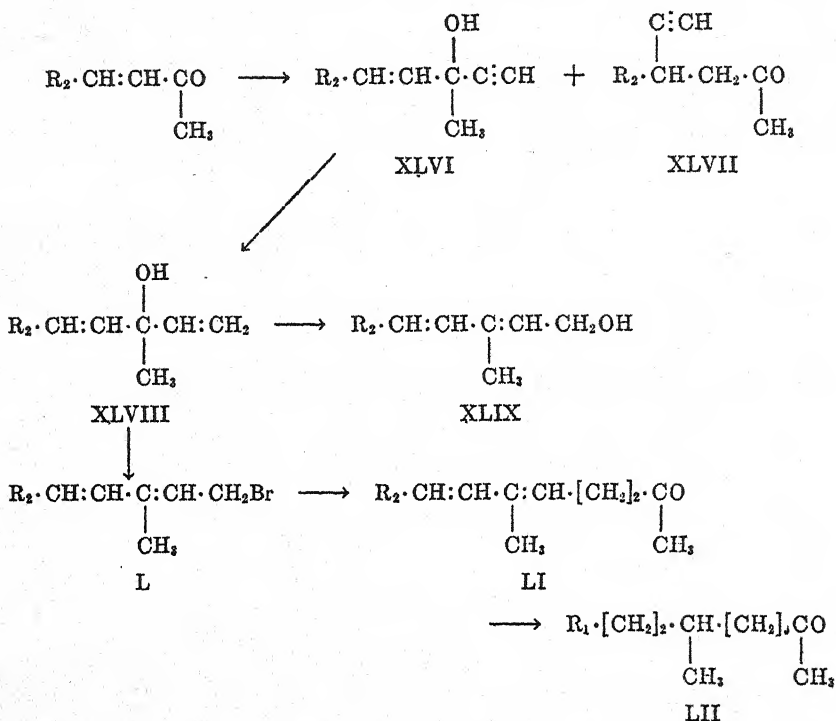
A further interesting observation with regard to this aldehyde has since been made by two of the present authors (185). β -Ionone has been found to condense readily with methyl cyanoacetate in acetic acid in the presence of acetamide [Cope (186)] giving the cyano-ester XLIII, which has been successfully hydrolyzed to the cyano-acid XLIV and decarboxylated to the nitrile XLV. Reduction of the latter with stannous chloride

by the method of Wittig and Kethur (187) gave a poor yield of an aldehyde which proved to be identical with that obtained above by distillation of the barium salt of the triethenoid acid.

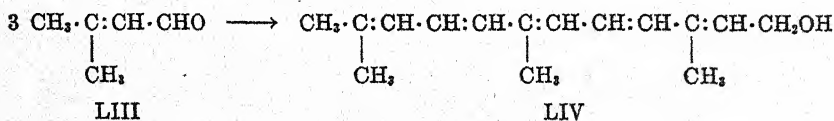


In 1935 Gould and Thompson (188) described preliminary work in connection with the proposed synthesis of a dihydro-vitamin A which they considered might possibly possess growth-promoting activity, dehydrogenation to the vitamin occurring *in vitro*. β -Ionone was condensed with acetylene in the presence of potassium tert. amyloxide giving a relatively poor yield of the acetylene carbinol, XLVI, together with the ketone XLVII and much polymerized material. The acetylene bond in the carbinol was successfully reduced to the ethylene (XLVIII) and the product re-arranged either directly with acetic anhydride to the primary alcohol, XLIX, or by means of phosphorus tribromide to the primary bromide, L. Condensation of the latter with ethyl sodio-acetoacetate and subsequent alkaline hydrolysis gave the C_{18} ketone, LI, which was readily hydrogenated to the perhydroketone. Proof that no change in the carbon skeleton had taken place during this series of reactions was supplied by the fact that the same saturated ketone was obtained by carrying out the above reactions starting from tetrahydro-ionone, the semicarbazones from the two preparations being identical. Furthermore, they had the same melting-point as that recorded by Karrer and Morf (177) for the derivative of the compound obtained by the method described in Section V. No further work on these lines has since been recorded.

The first claim to have synthesized vitamin A itself appeared in 1936. Fuson and Christ (189) found that by condensation of β -cyclocitral with β -methylcrotonaldehyde in the presence of the piperidine-acetic acid catalyst of Kuhn, Badstübner, and Grundmann (190), followed by reduction of the crude condensate with aluminum isopropoxide, a product was obtained showing an absorption band in the region of $328 \text{ m}\mu$ and giving with chloroformic antimony trichloride a blue color very similar to that



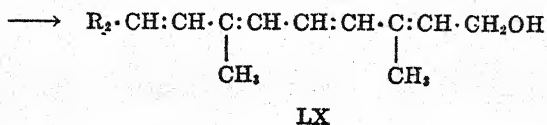
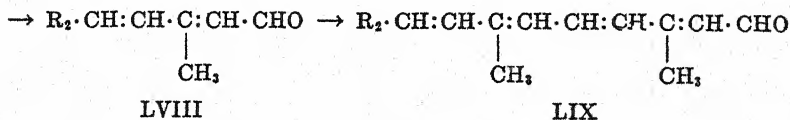
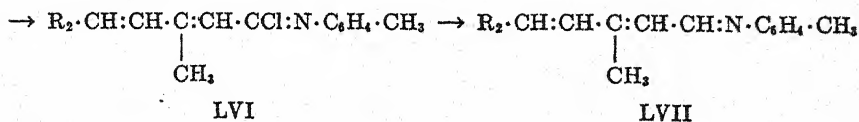
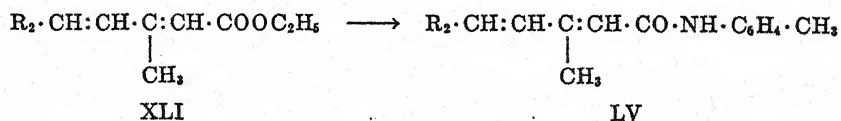
given by vitamin A. Heilbron and Jones (191), however, pointed out that these results could be accounted for by the presence of the pentaethenoid acyclic alcohol farnesinol, LIV, obtained from the condensation product of β -methylcrotonaldehyde with itself as described by Fischer and Hultzs (192).



The experiences of Heilbron and his colleagues indicated that it is impossible to condense β -cyclocitral with β -methylcrotonaldehyde under these conditions, a conclusion subsequently confirmed by Kuhn and Morris (193).

The first authenticated synthesis of a material displaying vitamin A activity was reported by Kuhn and Morris (193) in 1937 employing the following series of reactions. The ester XLI above, (178), was converted into the *o*-toluidide LV of the corresponding acid by reaction with the Grignard compound prepared from *o*-toluidine and methylmagnesium iodide, followed by treatment with phosphorus pentachloride in benzene

to give the chlor-imide LVI. Reduction of the latter with chromous chloride in ether by the method of von Braun and Rudolph (194) gave the *o*-tolil LVII of β -ionylidene-acetaldehyde, LVIII, from which the free aldehyde was obtained by distillation with steam in the presence of 10% oxalic acid. The aldehyde was a pale yellow somewhat unstable oil giving a reddish-brown precipitate with chloroformic antimony trichloride and characterized by a semicarbazone, m.p. 193–195°. The latter exhibited an absorption band of high intensity at 320 m μ [Kuhn (195)] a position in agreement with the aldehyde possessing three double bonds in conjugation with the carbonyl group. In contrast to β -cyclocitral, β -ionylidene-acetaldehyde was found to condense with β -methylcrotonaldehyde in the presence of the piperidine-acetic acid catalyst yielding a product which gave a blue-green color with antimony trichloride. On reduction with aluminum isopropoxide a mixture of alcohols was obtained giving with antimony trichloride a blue color showing an absorption maximum at 606 m μ , and after partial purification by adsorption on alumina a material was obtained which caused resumption of growth and cured xerophthalmia in rats reared on a vitamin A free diet. The order of activity of the purest specimen was given as 750 C.L.O. units which these authors calculated to correspond to a vitamin A content of approximately 7.5%, apparently on the basis that the concentrate of Karrer, Morf, and Schöpp (149), with a value of 10,500 C.L.O. units, was practically pure. In view of the latest values for crystalline vitamin A, however, the vitamin content of the synthetic material must have been considerably less than that claimed.

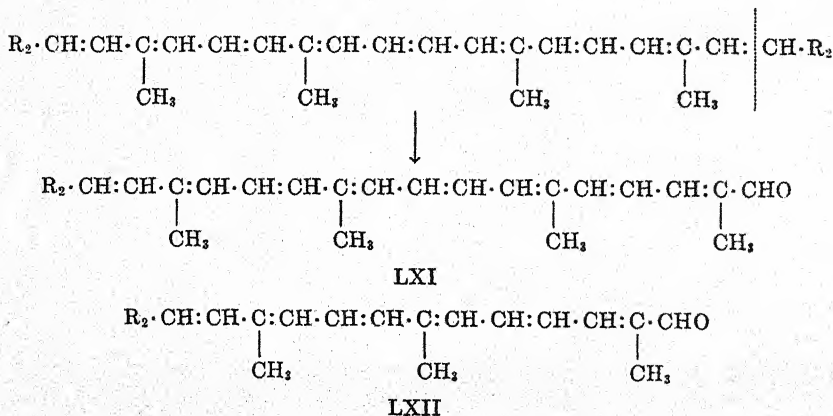


Attempts to repeat this synthesis have since been carried out by Karrer and R  gger (196) and by Krauze and Slobodin (197). The former authors state that condensation of β -ionylidene-acetaldehyde with β -methylcrotonaldehyde under the conditions described gives a product which on reduction with aluminum isopropoxide yields a mixture of polyenes. The main constituent is a compound which gives with antimony trichloride a color exhibiting an absorption maximum at 602 m μ . It is not, however, vitamin A since it can be separated from the latter chromatographically, but the possibility cannot be excluded that the complex mixture of which it is part contains vitamin A. Krauze and Slobodin (197), however, state that the crude reaction product is not biologically active since it does not prevent xerophthalmia in rats in doses as high as 40 γ daily.

VII. HOMOLOGUES OF VITAMIN A

1. Degradation of β -Carotene

In Section III it was shown that β -carotene is converted *in vivo* into vitamin A although there is some doubt whether this transformation can be effected *in vitro* by extracts of liver tissue. Attempts to imitate this reaction by chemical means have proved abortive, but recently Karrer and his colleagues have demonstrated that it is possible to degrade β -carotene to yield homologues of the vitamin. Thus Karrer and Solmssen (198) showed that by oxidation of a benzene solution of β -carotene with dilute aqueous potassium permanganate at room temperature and separation of the products by chromatographic adsorption, a crystalline aldehyde could be isolated (60 mg. from 700 mg. of carotene). From analytical and spectroscopic data for the aldehyde and its derivatives they established that it possessed the structure LXI, and is clearly formed by selective oxidation of one of the β -ionone rings.



For LXI Karrer, Solmssen, and Gugelmann (199) proposed the name β -apo-2-carotenal in order to indicate the position at which rupture of the chain has occurred. The necessity of some such nomenclature arose on the isolation by these investigators of a second homologue which they showed to possess the structure LXII, (β -apo-4-carotenal). In contrast to β -apo-2-carotenal, this aldehyde could not be obtained crystalline. As regards the biological activity of the two aldehydes and their oximes, spectroscopic data for which will be found in Table IV, good growth was obtained in vitamin-A-deficient rats with doses of 5 γ , 5 γ , and 20 γ respectively of β -apo-2-carotenal, β -apo-2-carotenal oxime, and β -apo-4-carotenal oxime. Subsequently, von Euler, Günther, Malmberg, and Karrer (200) showed that the liver oils of rats depleted of vitamin A and then fed on massive doses (50 γ) of β -apo-2-carotenal exhibit an inflexion

TABLE IV

M.p. and Spectroscopic Data of Apocarotenals, their Oximes, and Apocarotenols

	M.p.	λ_{\max} (CS ₂) m μ	λ_{\max} (Pet. Ether) m μ	λ_{\max} (EtOH) m μ
1. β -Apo-2-carotenal.....	139°	525, 490, (458)	484, 454	Indefinite
2. β -Apo-4-carotenal.....	—	460 (indef.)	442 (indef.)	—
3. α -Apo-2-carotenal.....	158°	519, 484, 454	479, 450	Indefinite
4. Oxime of (1).....	180°	507, 473	471, 441	475, 445
5. Oxime of (2).....	165°	456	408	409
6. Oxime of (3).....	178°	499, 469	466, 438	469, 439
7. β -Apo-2-carotenol.....	145°	486, 456	453, 423	456, 426
8. α -Apo-2-carotenol.....	157°	478, 448	446, 420	448, 423

at 320 to 330 m μ indicating that the aldehyde, like β -carotene, is converted into vitamin A in the body.

A similar oxidation of α -carotene by von Euler, Karrer, and Solmssen (201) gave an aldehyde which, from analytical and spectroscopic data and the fact that it was inactive in doses of 20 γ , they formulated as LXIII, (α -apo-2-carotenal), the β -ionone ring having apparently been preferentially oxidized.

The reduction of LXI and LXIII with aluminum isopropoxide to β -apo-2-carotenol and α -apo-2-carotenol respectively was investigated by von Euler, Karrer, and Solmssen (201). Both products were crystalline solids (see Table IV) giving blue colors with chloroformic antimony trichloride, but β -apo-4-carotenol obtained in a similar manner was a viscous oil. A secondary alcohol, LXIV, which was very similar spectroscopically to β -apo-2-carotenol was subsequently prepared by Karrer, Rüegger, and Geiger (202) by condensation of β -apo-2-carotenal with ethyl magnesium bromide,

hand two of the present authors (I.M.H. and W.E.J.) have encountered no difficulty in effecting this reduction, the alcohol so obtained exhibiting a single well-defined absorption band of high extinction at $359\text{ m}\mu$ and giving with antimony trichloride a blue color with a single band at $712\text{ m}\mu$.

3. Vitamin A_2

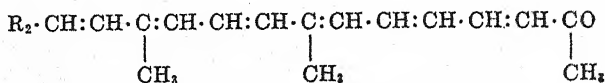
Interest in the chromogen responsible for the $693\text{ m}\mu$ band, already mentioned as being sometimes present in the antimony trichloride blue color, was stimulated by two simultaneous independent observations, *viz.*:

(a) Edisbury, Morton and Simpkins (206) observed that whereas in halibut liver oils the ratio of the $620:693\text{ m}\mu$ intensities is generally of the order 6:1 and in halibut visceral oils 10:1, the $693\text{ m}\mu$ band is rarely detectable in cod liver oils and never in whale liver oils. In goldfish eyes, however, they found the ratio to be about 1:1.5, and in the unsaponifiable matter from the liver and viscera of brown trout only the $693\text{ m}\mu$ band could be detected. In the latter case the ultra-violet absorption spectrum showed bands at 470 , 350 , and $287\text{ m}\mu$ and these authors, taking into account its apparent physiological similarity to vitamin A (Wald, 206a), tentatively named the $693\text{ m}\mu$ chromogen vitamin A_2 .

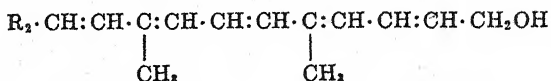
(b) Lederer and Rosanova (207) found that liver oils from certain Russian fresh-water fish gave a stronger 693 than $620\text{ m}\mu$ band. These results were subsequently confirmed in a preliminary report by Lederer, Rosanova, Gillam, and Heilbron (208) and in addition it was observed that in these oils the absorption spectra are differentiated from those of marine fish liver oils in that the maximum is displaced from $328\text{ m}\mu$ to $345\text{--}350\text{ m}\mu$ with the frequent appearance of another band at $280\text{--}285\text{ m}\mu$. In a more detailed investigation of these oils, Gillam, Heilbron, Jones, and Lederer (209) found that the average $693:620$ intensity ratios are generally of the order 2:1 in contrast to the ratio of 0.15:1 for the corresponding oils from marine fish, similar results being obtained for the unsaponifiable concentrates.

With a view to determining the constitution of this chromogen a concentrate having the following values:— $E_{1\text{cm}}^{1\%} 697\text{ m}\mu = 2600$, $620\text{ m}\mu = 1,200$, $344\text{ m}\mu = 850$, was subjected to chromatographic analysis and distillation in high vacuum, but in neither case could any increase in the $693:620$ intensity ratio be recorded. Since the two chromogens appeared to be very similar it seemed probable that there might be a simple relationship between their two structures, and in view of the absorption maximum at $345\text{--}350\text{ m}\mu$ it was accepted as a working hypothesis that the new chromogen contained a system of six conjugated double bonds. Proof that it contains a β -ionone ring was supplied by the fact that ozonolysis gave geronic acid, the amount of the latter isolated being approximately

the same as that from a halibut liver oil concentrate of similar blue value. Oxidation with aluminum tert. butoxide in the same manner as described above for vitamin A gave a ketone $C_{25}H_{34}O$ (*p*-chlorobenzoylhydrazone, m.p. 142–143°) which by analogy with the product from the classical vitamin A, probably possesses the structure LXVI, vitamin A_2 therefore being LXVII.



LXVI



LXVII

On the other hand, Gray and Cawley (211) from a study of its elimination curve concluded that it probably contains the same number of carbon atoms but one double bond more than vitamin A, while Embree and Shantz (212) showed that the products obtained on cyclization of the two compounds with alcoholic hydrochloric acid have very similar absorption spectra. Karrer, Geiger, and Bretscher (374) have suggested that vitamin A_2 is related to lycopene as vitamin A is to β -carotene.

As regards the growth-promoting activity of this chromogen, considerable difficulty has been encountered in its estimation owing to contamination with vitamin A, but from a comparison of the prophylactic activity of two concentrates of equal blue values from marine and fresh-water fish liver oils, the above authors concluded that it probably did possess vitamin A activity. This was subsequently confirmed by Gillam (210) and by Lederer and Rathmann (213) who also showed that another band at 645–650 $m\mu$ accompanies that at 693 $m\mu$ in the antimony trichloride color given by fresh-water oils. They suggest that this bears the same relationship to the 693 $m\mu$ band as the 583 bears to the 617 $m\mu$ band in the color obtained with marine liver oil concentrates. This result indicates that in previous estimations by the blue color the intensity found for the maximum at 620 $m\mu$ was actually too high owing to absorption at 645–650 $m\mu$, thus further justifying the title 'vitamin A_2 .'

Recently Morton and Creed (214) have shown that carotene behaves as a provitamin A_2 for perch and dace, and further observations on the relative distributions of vitamins A and A_2 have been recorded by Lovern, Morton, and Ireland (215), Lovern and Morton (216), Wald (217), and Lederer and Verrier (218).

VIII. THE MEASUREMENT AND DISTRIBUTION OF VITAMIN A

1. *Difficulties of Biological Assay*

Chemical work on vitamin A depends on the establishment of biological activity. Chemical and physico-chemical tests are of value only in so far as they show high correlation with the characteristic physiological properties of the vitamin. In some investigations it is permissible to dispense with biological checks; for example, in the preparation of vitamin A concentrates by extraction from fish liver oils, the distribution of the vitamin can be adequately checked, for most purposes, by determination of the ultra-violet absorption spectrum or even by use of the antimony trichloride test. If, however, there has been serious decomposition, nothing but a biological assay can establish whether or not any physiologically inert decomposition products are simulating vitamin A.

Early work on vitamin A was seriously handicapped by the usual difficulties of biological technique. These difficulties have been particularly great with the vitamins, because it has often been impossible to know whether the responses to doses of a concentrate or synthetic material have been limited by the absence from the animals' diets of other essential ingredients. Many of the inconsistencies apparent in earlier work have been resolved by the subsequent increase of knowledge, and it is not proposed here to give any detailed account of early animal experiments.

2. *International Unit and Standard Preparation*

At the present day the unit of vitamin A has been fixed by the Permanent Commission on Biological Standardization of the Health Organization of the League of Nations (219) as the activity of a definite weight, 0.6 $\mu\text{g.}$, of a specimen of purified β -carotene. This, dissolved in a vegetable oil at such concentration that 1 g. contains 0.3 mg. of β -carotene, constitutes the International Standard Preparation of vitamin A. One International Unit of vitamin A is therefore contained in every 2 mg. of the International Standard Preparation.

Correct use of the International Standard Preparation involves comparing the biological effect of a known amount with that of a known amount of the substance whose vitamin A content is under investigation. In accordance with its general procedure, the Permanent Commission has not laid down methods of test, but the report of the Second Conference on Vitamin Standardization (219) indicates that the restoration of growth following administration of vitamin A or the cure or prevention of xerophthalmia may be used as criteria of vitamin A activity.

3. *Criteria of Response to Vitamin A*

The most commonly used criterion of response to vitamin A is the rate of growth (*i.e.* increase in body-weight) of young animals that have been fed

exclusively on a vitamin-A-free diet for sufficiently long to have remained stationary in weight over an adequate period. With big enough groups of animals the average increase in weight should be a function of the amount of vitamin A given to supplement the basal diet, of which a very large number of variations have been described in the literature. Reference may usefully be made to the survey by Bomskov (220). Coward (221) has described particular procedures in some detail and similar descriptions will also be found in the *Pharmacopoeias* (222, 223). A modification of the usual procedure, wherein increase in weight is the metameter of measurement, has been suggested by Sherman and Todhunter (224). Their procedure would seem to have advantages in economy of time and material.

It has been maintained [cf. Williams (225)] that weight increase is an undesirable criterion of vitamin activity because of its non-specific nature and indirect relationship with deficiency of the vitamin. It is, however, pertinent to point out that none of the criteria that have from time to time been suggested as a basis for vitamin A testing are in this respect to be qualitatively distinguished from weight increase. The characteristic eye infection, xerophthalmia, is itself only an indirect manifestation of vitamin A deficiency, following upon failure of the epithelial mucosa surrounding the eye to maintain a healthy condition. The macroscopic symptoms of xerophthalmia are the result of an infection, probably of organisms that are not pathogenic to the healthy animal. Its use as a criterion, along with changes in body-weight, has been described by Scheunert and Schieblich (226), but, it may be noted, Coward (221) no longer considers this a satisfactory basis, although it was originally proposed by Steenbock and herself some years ago (227).

The metaplasia of epithelial mucosa in general is characteristic of vitamin A deficiency. One manifestation thereof is the appearance of cornified cells in the vagina of the young female rat, detected by the usual process of examining vaginal "smears," a condition that was originally described by Evans and Bishop (228). It has been studied by a number of authors (229, 230, 231, 232). The position was reviewed by Baumann and Steenbock (233) who suggested that the condition might be made the basis of an assay method, and Moll, Dalmer, Dobeneck, Domagk, and Laquer (234) used it in a "single dose" method for estimating vitamin A in various materials; Coward, Cambden, and Lee (235), however, after studying this proposed method statistically, concluded that its error was greater than that of the rat-growth method. Moreover, the vaginal manifestation of vitamin A deficiency is certainly not specific. It is a regular feature of estrus in the normal mature rat and can be produced in the immature and in the ovariectomized female rat by the administration of suitable hormones.

Xerophthalmia and vaginal cornification have, further, an additional dis-

advantage as criteria in quantitative evaluations in that they constitute the kind of response known as "all or nothing" or quantal. In order to measure by their means the effects of different doses of a test material or a standard, it is necessary to use groups of animals and to determine the percentage of response within the group. This method is inherently less accurate than a method based upon graded responses; moreover, there is always a subjective element in judging whether a response is positive or not. These difficulties may be partly overcome by using as the metameter of measurement the time in days taken to cure an animal of the particular manifestation of vitamin A deficiency, such as xerophthalmia or kolpokeratosis. This procedure is still open to the criticism that the decision whether or not the marginal animal shows the condition is a matter of individual opinion and is itself not a measurable quantity. The matter has been critically discussed by Coward (236).

4. Error of Biological Assay

Apart altogether from general and theoretical considerations, an assay method is to be judged by its probable limits of error, which can be determined by statistical methods from the figures obtained in the test itself, when this is properly designed. Coward's conclusions, based upon statistical analysis, are borne out in practice by the fact that the majority of workers in this field have used and continue to use growth response as a criterion of vitamin A activity. It is of course essential that there shall be no limiting factor in the basal diet of the experimental animals, other than vitamin A itself. Provided the response shown to optimal quantities of vitamin A is not to be obtained with any other known substance, and provided this optimal response results in a growth-rate not significantly different from that of normal animals on a full stock diet, the essential conditions for a satisfactory growth test are secured.

The probable limits of error of the test most commonly employed, the rat-growth test, were studied by a sub-committee of the British Pharmacopoeia Commission (237). They found that tests with 40 rats might once in twenty times give below 52 or above 193 per cent. of the true value, suggesting a somewhat higher probable error than for other vitamin tests. It has, however, been claimed by Wilkinson (238) that routine tests may be carried out with a lower error.

Many of the difficulties apparently inherent in estimating vitamin A by the curative method are to be attributed, according to Richards and Simpson (239), to the sick conditions of the experimental animals at the time they are judged to be "run out" of vitamin A, which may be anything from four to ten weeks after weaning on to a vitamin-A-free diet. The conditions of even those rats that had been given "curative" doses of the vitamin

witness in a striking manner the wide-spread and lasting damage associated with avitaminosis A; the matter is referred to again in Section IX, 2. Coward (240), however, claims that the variance in the growth-response of animals used in the curative test for vitamin A is no greater than the variance in growth of stock animals on a normal diet. However that may be, there is no evidence from the published literature that a prophylactic method is likely to replace the customary curative procedure, although this would be expected to eliminate some of the irregularities of response attributed to the sick condition of the test animal. Bomskov (241) states that the prophylactic procedure gives satisfactory results but cites no published evidence in support; Irving and Richards (245) suggest the use of a prophylactic technique based on histological assessment of degeneration in the central nervous system.

There is a special difficulty in vitamin A testing, arising from the nature of the International Standard Preparation. At the time when this was chosen, the opinion was held, and held on good evidence, that a purified stable source of vitamin A was not available in sufficient quantities to make a practical standard. Purified carotene, on the other hand, was known to be relatively stable even in oil solution, and its ability completely to replace vitamin A in the diets of rats deprived thereof was fully established, as mentioned earlier in this review. Morgan, Edisbury, and Morton (242) have pointed out that the validity of the existing International Standard Preparation depends upon the assumption that all experimental animals at all times are equally efficient, not only in making use of the resultant vitamin A, but also in converting β -carotene into vitamin A. For this there is no direct evidence and the assumption introduces a complication into attempts to evaluate vitamin A biologically in terms of β -carotene.

The claim by Moll and Reid (244) to have found esterified vitamin A approximately twice as biologically active as the alcohol has not been substantiated by any other workers.

5. Biological Activity, Ultra-violet Absorption, and "Blue Value"

On the basis of the purified specimens of vitamin A available at the time when β -carotene was chosen for the International Standard Preparation, and of further evidence obtained in the examination of fish liver oils, a relationship was established between the absorption coefficient of vitamin A and its biological activity. A provisional factor of 1600 was laid down by the Permanent Commission (219) and it was made permissible to convert the value for $E_{1\%}^{1\text{cm}}$ 328 $m\mu$ to international units per gram by use of this factor. Thus, an oil having an absorption coefficient of 0.5, as determined on the unsaponifiable matter of the oil, would be assayed at 800 International Units of vitamin A per gram. For richer oils saponification is frequently

dispensed with; a halibut oil with an absorption coefficient of 20, as determined on the oil, would be assessed at 32,000 International Units per gram. The relationship between colorimetric and spectroscopic tests, and the relationship of both with the biological method, have been discussed in detail by the Medical Research Council of Great Britain (243).

The validity of the factor, even for highly purified concentrates of vitamin A, depends on two determinations: first, the measurement of ultra-violet absorption, which can be conducted with a high degree of accuracy; secondly, biological assay which, as already pointed out, has a large inherent error. Attempts have more than once been made to establish by collaborative tests the best value for the factor, but so far such tests have not been held to justify any change in the provisionally recommended factor.

The recent work of Holmes and Corbet (158) and of Mead (165), and Coward and Underhill (180) points to a value of 3.0 to 3.2×10^6 International Units per gram of vitamin A in the pure state, and also to a value for the extinction coefficient at the ultra-violet absorption maximum of $E_{1\text{cm}}^{1\%}$ 2,000–2,100. The reference cod liver oil used however in the United States as a subsidiary standard would appear to have an abnormally high conversion factor. Various workers, *e.g.* Ward and Haines (246) and Emmett and Bird (247) (*cf.* also 248) have claimed for it a conversion factor of 2,000 or over, on the basis of its stated biological activity (3,000 International Units per gram) and its ultra-violet absorption ($E_{1\text{cm}}^{1\%} = 1.5$ or under). Recent investigations by Hume (249) suggest that the biological activity is actually nearer 2,600 International Units per gram, but even this value involves a conversion factor of 1,750, rather than 1,600 (*cf.* Robinson, 250).

As has already been discussed in this review, the intensity of the color produced with antimony trichloride, especially if the test is carried out spectrophotometrically on the unsaponifiable matter of the oil, shows a high degree of correlation with the ultra-violet absorption. Conversion of the "blue value" to International Units per gram might, therefore, be thought to be a legitimate procedure, but this is not in accordance with the best practice. There are inherent difficulties, as discussed by Notevarg and Weedon (39, 48) in measuring the intensity of the blue color, and this is partly the reason why various workers, *e.g.* Lathbury (251) and van Eekelen, Emmerie, and Wolff (252) have put forward different factors for converting "blue values" into International Units per gram. For routine purposes, especially if the tests are conducted under identical conditions by the same operator, it is possible to define those conditions sufficiently closely to make the color-reaction very useful for control purposes. When the test has been carried out by a suitable modification of the original Carr-Price procedure, then a factor of 30 has been found suitable for converting the "blue value"

into International Units per gram. Some of the objections to this conversion disappear if the color is evaluated spectroscopically, but the test then loses much of its simplicity and therefore of its utility, and appears to possess little advantage over the direct measurement of ultra-violet absorption.

6. Vitamin A and Carotene Contents of Dairy and Other Products

Determination of the vitamin A value of milk-fat by other than biological methods presents, as shown by Gillam (253), a peculiar difficulty owing to the concomitant presence of carotene. Physico-chemical methods for the separation of vitamin A from carotene are open to the objection that some of either or both active substances may be lost during the process; attempts to correct for carotene by measurement of the characteristic absorption in the visible spectrum may be frustrated by the presence of biologically inactive carotenoids such as xanthophyll. A study by Moon (254) suggests that previously published figures for the carotene:xanthophyll ratio of grass and other plants should possibly be accepted with some reserve. Values for carotene and xanthophyll ratios in vegetables and in farm and dairy products have been published by several authors (255, 256, 257, 258, 259). Even when xanthophyll and carotene have been effectively separated, the further difficulty remains that α -, β - and γ -carotenes have different biological activities, the first and the last being half as effective as the other. Some workers, *e.g.* Booth, Kon, and Gillam (260), Hilton, Hauge, and Wilbur (261), and Guerrant and Dutcher (262) have, therefore, preferred to assess the total vitamin activity of dairy products by determining it biologically.

In an investigation of the colostrum from various breeds of cows, Gillam, Heilbron, Ferguson, and Watson (257) demonstrated that, in comparison with ordinary milk, it is characterized by greatly increased contents of both carotene and vitamin A (see Table V), thus confirming the earlier work of Dann (263) and Semb, Baumann, and Steenbock (264). According to van Eekelen and de Haas (265) the same relationship holds for human colostrum and human milk. Stewart and McCallum (267, 268, 269) have found that the vitamin A potency of bovine colostrum, which is high but very variable, is not influenced by differences in dietary, breed, or date of calving, and cannot be further raised by feeding up to 3 lbs. of carrots per animal per day for 2 to 6 months.

It is to be regretted that there is still to be found in the literature a considerable amount of unsatisfactory information about the results of biological tests for vitamin A. Although vitamin A itself has never been found in vegetable products, many writers continue to talk of carotene and vitamin A as synonymous. This would, in itself, not be so serious if the vitamin A activity of vegetable products were always expressed in International Units, as the result of a contemporaneous comparison with the International

Standard Preparation or a suitable subsidiary standard. But even this essential procedure is sometimes ignored, and the results are expressed in terms of some animal "unit", which is more an expression of the sensitivity of the animals used than of the vitamin A content of the test material. Coward, Key, and Morgan (266) have shown that, within the same laboratory and with the same stock of animals, the response to the vitamin A in cod liver oil may show a five-fold range; it is precisely in order to eliminate the disturbing effect of this variation that Standard Preparations have been made available for use contemporaneously with the test material on strictly comparable groups of animals. If these groups are sufficiently large, the variations within the groups receiving Standard and test materials will be

TABLE V
Carotene and Vitamin A in Colostrum from Various Breeds of Cows

Breed	Date of sample	Carotene (mg. % fat)	Vitamin A (mg. % fat)
Friesian	Oct. 20	3.40	1.24
	Nov. 20	0.38	0.52
Ayrshire	Nov. 22	4.60	2.96
	Dec. 22	0.18	0.37
Guernsey	Oct. 28	3.61	1.13
	Nov. 28	0.91	0.67
Shorthorn	Oct. 13	2.90	3.51
	Nov. 13	0.28	0.48

of the same order, and deductions based on the average response will partly eliminate errors due to variations in animal sensitivity.

7. Vitamin A Contents of Foods and Tissues

From what has been written earlier in this review, it will have become clear by implication that fish liver oils constitute the richest known source of vitamin A. According to the tables of vitamin contents compiled by Fixsen and Roscoe (270) and by Daniel and Munsell (271) values as high as 500,000 International Units per gram have been reported. Such figures, however, must be regarded as quite exceptional and the result of indefatigable commercial prospecting. The best known of all medicaments containing vitamin A is cod liver oil, and oils of good medicinal quality generally contain between 1,000 and 2,000 International Units per gram.

As has already been pointed out, the vitamin A activity of dairy products is partly due to their carotene content; if the total activity is expressed in

terms of vitamin A itself, the fat of milk from cows that have been fed on fresh pasture may be as high as 50 International Units per gram, though lower values are more usual. Butter-fat with this activity will confer on

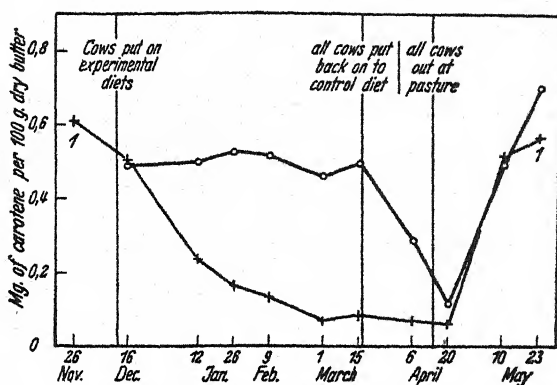


Fig. 1. Carotene of Butters

+ — +, control ration. O — O, artificially dried grass ration

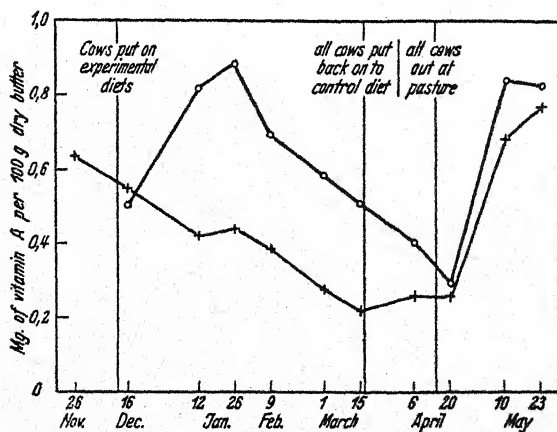


Fig. 2. Vitamins of Butters

+ — +, control ration. O — O, artificially dried grass ration

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the milk a vitamin content of 2 International Units per gram, or about 1,000 International Units per pint.

Interesting observations regarding the carotene and vitamin A contents of butter have been made by Gillam, Heilbron, Ferguson, and Watson (257) who found that, by substituting dried grass for part of the concentrates usually given to stall-fed cattle in winter, both the vitamin A and the

carotene content of the butter could be maintained at nearly the same level as that obtained when the cows were fed on fresh pasture. The results are strikingly demonstrated in Figs. 1 and 2.

Apart from fish liver oils and milk fat, the chief ordinary sources of vitamin A activity are the green leaves of plants, and root crops, such as carrot, which contain carotene. Thus the cabbage contains about 9 International Units per gram and the carrot about 20. An unusually high figure has been

TABLE VI
*The Vitamin A and Carotene Contents of some Important Sources**

Sources	Vitamin A	Carotene	Total Vitamin A Activity
	<i>in mg. per 100 g.</i>	<i>in mg. per 100 g.</i>	<i>in I.U. per g.</i>
Butter (milk-fat).....	0.3 to 1.5	0.1 to 2	10 to 60
Cabbage, green leaves (on dry weight).....	none	16	(300)
Carrot.....	none	2 to 10	(35 to 200)
Egg yolk, hens'.....	(3 to 4)	0.1 to 4	90
Grasses, various (on dry weight).....	none	40 to 70	(700 to 1,200)
Liver (calf).....	(20 to 60)	prob. small amount	500 to 1,600
Liver oil, cod.....	(16 to 160)	none	400 to 4,000
Liver oil, halibut.....	(800 to 15,000)	none	20,000 to 360,000
Milk, cows'.....	(0.01 to 0.05)	(0.003 to 0.06)	(0.3 to 2)
Milk, human.....			1.5 to 2
Palm oil, red.....	none	24 to 300	(350 to 5,000)
Spinach.....	none	3 to 7	(50 to 150)
Tomato (ripe flesh).....	none	14 to 36	(300 to 700)

* Adapted with acknowledgements from Boas-Fixsen and Roscoe's Tables (270). Figures in brackets have been calculated. Figures for "Vitamin A in mg. per 100 g." have been obtained spectroscopically by means of the factor 1600, or by calculation, assuming 1 mg. of "pure" Vitamin A to contain 2,600 International Units. Figures for "Total Vitamin A activity" have been obtained by calculation or by biological test.

recorded for tinned strained spinach, while among vegetable oils red palm oil is exceptional in containing carotene equivalent to nearly 2,000 International Units per gram. Whole wheat contains no biologically active carotenoids [Zechmeister and Cholnoky (371), Zechmeister and Escue (372)].

A list of the commoner sources of carotene and vitamin A is given in Table VI.

Although mammalian and avian liver oils are in general less rich sources of vitamin A than most fish liver oils, their contents help to establish the fact

that the liver is the probable store of vitamin A in the vertebrate organism. The actual method of conversion of carotene into vitamin A has not been ascertained. There is some evidence, adduced by Drummond, Gilding, and MacWalter (272) that the reticulo-endothelial cells of the liver are involved in vitamin A metabolism. Both vitamin A and carotene are present in the blood of animals (including man) receiving adequate quantities, and work by Lawrie, McArdle, and Moore (273) has pointed to its excretion in the urine, at any rate in certain pathological conditions.

The placental passage of vitamin A from mother to offspring has been studied by Dann (274), who finds a physiological barrier above a certain level. Increases in the maternal intake of vitamin A are accompanied by progressively smaller outputs in the milk, until no further enrichment of the milk can be brought about.

The correlation of infectivity with liver reserves of vitamin A, as estimated by the antimony trichloride test, has been extensively investigated by Moore (275), Wolff (276) and others.

A recent curious finding (Jonsson, Obel, and Sjoberg, 278) is that the rat, which is independent of exogenous ascorbic acid, loses its ability to synthesize vitamin C, or to make use of dietary vitamin C, when made completely deficient in vitamin A. The effect of certain carcinogenic compounds in lowering hepatic stores of vitamin A is also a finding of interest (279, 280, 281).

IX. THE PHYSIOLOGICAL ACTION OF VITAMIN A

1. *Vitamin A Deficiency*

While shortage of thiamin, nicotinic acid, ascorbic acid, and vitamin D are associated with mild or severe symptoms of beri-beri, pellagra, scurvy, or rickets, respectively, there is no such clear-cut association of vitamin A deficiency with a recognized clinical syndrome. Before considering some of the specific clinical and pathological manifestations of vitamin A deficiency, reference may be made here to certain features of a more general nature. Though a reduction in growth-rate, to the point of complete cessation, is invariable in experimental animals receiving markedly sub-optimal intakes of vitamin A, direct evidence of such an effect in the growing human being does not appear to have been recorded; in any event, it would be difficult to obtain. Again, characteristic effects on the reproductive processes of male and female rats have been recorded by various groups of workers, of whom may be mentioned Evans (277), Sure (282), Sampson and Korenchevsky (283), and Evans (284); in particular the histological studies of Mason (285, 286) have led to a clear differentiation from the superficially similar effects of vitamin E deficiency. Indeed, this author (287) has demonstrated the possibility, by examination of the uterine contents of rats at various

stages of pregnancy, of distinguishing the different effects of a simultaneous deficiency of the two vitamins.

The relationship between vitamins A and E has been studied by a number of authors, following Moore's finding (373, *cf.* also 288) that vitamin-E-deficient animals have unusually low reserves of vitamin A in their livers. It is also now recognized that the muscular dystrophy produced in rabbits by vitamin E deficiency is identical with that due to rancid diets or diets containing cod liver oil (Mattill and Golumbic, 289). Hickman, Harris, and Woodside (290) claim that limiting quantities of vitamin A or carotene are used more effectively in the presence of vitamin E than in its absence. At present it is probably not justifiable to say more than that these relationships are probably related to the high anti-oxidant activity, and consequent easy oxidizability, of vitamin E.

2. Vitamin A and Infection

Cramer (291) was among the first to observe general ill-health, accompanied by increased liability to general infection, in animals deprived of vitamin A. The association of this infectivity with damage to epithelial tissues, especially to epithelial mucosa as in the respiratory and genito-urinary tracts, has been established by a large number of workers, on a variety of animal species. Attempts to show any effect of the deficiency on the normal immunity mechanism of the body have not been successful, although Osborn (292) claims to have detected some rise in hemolytic complement as a result of feeding cod liver oil, and a lowering of the complement in rats receiving insufficient vitamin A. That epithelial metaplasia is associated with all stages of vitamin A deficiency has been established by the work of many investigators (293, 294, 295, 296, 297). Green and Mellanby (298, 299) were so impressed with the relation between vitamin A deficiency and lessened resistance to bacterial invasion of the tissues that they suggested the descriptive adjective "anti-infective" for vitamin A. Mellanby and Green (300), and Green, Pindar, Davis, and Mellanby (301) were also led to investigate its use in the treatment of puerperal septicemia and the prevention of puerperal sepsis. The work of McCarrison (302) suggested that vitamin A deficiency in rats was connected with thyroid disturbances as well as with epithelial changes in the tracheal mucosa.

Following on Mellanby's work many authors have attempted to assess the effect on human infections of treatment with vitamin A. There is an extensive medical literature on the subject, ranging from individual case reports to statistical treatment of "mass" experiments, and covering such a variety of conditions as *otitis media* complicating scarlet fever (Sutcliff, Place, and Segool, 303), the common cold (Sherman, 304, and Shibley and Spies, 305), senile vaginitis (Simpson and Mason, 306), the sequelae of

measles (Ellison, 307), and respiratory infections in infants (Barenberg and Lewis, 308). It is hardly to be expected that any unanimity of opinion would result from such varied and difficult experimental procedures; nevertheless, the importance of vitamin A supplies for the maintenance of normal mucosa, the "first line of defense" of the body against invasion by micro-organisms, seems now to be almost universally accepted, in spite of conflicting reports as to the efficacy of vitamin A therapy, preventive or curative, against specific infections. As might be anticipated on theoretical grounds, the incidence of certain infections, such as the common cold (a virus disease), is not affected by increasing the vitamin A intake of "normal" populations, but there is an accumulation of data suggesting that the duration of these infections may be materially lessened by such treatment. This is presumably due to a reduced incidence of the usual sequelae, due to post-viral infections of bacterial origin taking effect through the mucosal epithelia weakened or damaged by the virus infection.

3. *Vitamin A and Vision*

The development of xerophthalmia in rats deprived of vitamin A is so evident and, in complete deficiency, so regular that a curious confusion has arisen. The close relationship between vitamin A metabolism and certain visual processes, specifically those of adaption to dim light, is one of the most clearly established facts in vitamin physiology. This has led certain authors to write as if xerophthalmia and night-blindness were both manifestations of the same immediate ocular disturbances (*cf.* Eddy and Dalldorf, 309). Although both conditions may arise, one directly and one indirectly from the same nutritional defect, they are entirely different. Xerophthalmia is very rare among human subjects and only a few cases are recorded (*cf.* Thorson, 310, and May and Wolff, 311) as having been subjected to vitamin A therapy; reference must also be made to an important study by Blegvad (312). Xerophthalmia is essentially a manifestation of severe deficiency and is difficult to produce with certainty even in experimental animals. It can be regarded as a secondary manifestation of a more general epithelial metaplasia, quite comparable with the infectious processes, abscesses, and so on, found widely distributed in animals and patients showing marked hypovitaminosis A. Where it exists, however, because of the severity of the causative deficiency, night-blindness is almost certain to be present to some degree as well. But here the causes are more direct and fundamental; as May and Wolff (311) imply, the retinal rods and cones are actually a particularly sensitive epithelial structure.

The association of night-blindness with vitamin A shortage is now seen clearly to be the explanation for the traditional treatment of the condition (*cf.* Mar and Read, 313)—with liver for example—and for its appearance in

severe forms when the butter of a population's diet had been completely replaced by unvitaminized margarine (314). Such outbreaks of severe night-blindness occurred some years ago and it is likely that the same dietary change would today be followed by the same results. With the addition of adequate amounts of vitamins A and D to margarine, however, there need be nothing to choose between it and summer butter as sources of the fat-soluble vitamins.

Systematic treatment of frank night-blindness with vitamin A has never been possible on an extensive scale, even after recognition of the condition as a true avitaminosis, owing to the relative scarcity of the disease at the acute stages. Spence (315) has studied the condition in a group of children, and cases have been reported by Aykroyd (316), Fisher (317), and Vaillant and Gillis (318). A similar condition has been produced experimentally in animals by Yudkin (319) and it has even been proposed (*cf.* Pletnjev, 320) to use the relative visual impotence of avitaminotic chicks as the basis of a quantitative test for vitamin A.

Several different investigators seem, at the beginning of the last decade, to have conceived independently the idea of testing for sub-clinical manifestations of night-blindness. The pioneer work of Jeans and Zentmire (321) appears to have been the first published; it introduced the biophotometer as a means of measuring degrees of hypovitaminosis and of following recovery under treatment. In spite of at least two papers, by Palmer and Blumberg (322) and Isaacs, Jung, and Ivy (323), expressing criticism and scepticism as to the value of the results obtained by Jeans and Zentmire (325) and Jeghers (324, 326) by biophotometry, a large amount of published data (327, 328) seems definitely to have established the procedure as trustworthy when properly controlled, and reasonably easy to carry out under normal conditions of clinical investigation. The already considerable literature of the subject has been thoroughly reviewed by Clausen (329), and a simplification of existing methods has recently been put forward by Pett (330). Of particular interest is a paper by Ezickson and Feldman (331) in which they establish a high degree of correlation between lowered dark adaptation and another manifestation of vitamin A deficiency, lithiasis, referred to in the next section.

The group of workers at the Dunn Nutritional Laboratories, Cambridge, England, have been particularly assiduous in standardizing technique for measuring dark adaptation and for correlating the results of standardized tests with the status of vitamin A nutrition. Harris and Abbasy (368) and also Yudkin (369) seem to have clearly established the claim that such tests, properly carried out and critically interpreted, can in fact provide a trustworthy indication of "preclinical" hypovitaminosis A. These views are shared by Hecht and Mandelbaum (370) among others.

The fundamental relationship connecting the metabolism of vitamin A and related carotenoids with the biochemical processes in the retinal rods and cones has been the subject of intensive research, especially by the groups of workers associated with Hecht and with Wald in U.S.A., and with K. Tansley and the late R. J. Lythgoe in England. Excellent reviews of the position have been given by Tansley (332) and more recently by Wald (333), the latter's survey including a good bibliography, to which reference should be made, as it is not proposed here to do more than mention the salient facts that have received general agreement.

The breakdown and regeneration of visual purple (rhodopsin) in the retinal rods are essential stages in the processes of dark adaptation taking place in the eyes of vertebrates. In mammals, birds, other terrestrial vertebrates, and predominantly marine fishes, vitamin A and related carotenoids constitute essential parts of the pigment concerned and its metabolites; in fresh-water vertebrates (including fish) vitamin A is partly or wholly replaced by "vitamin A₂" and rhodopsin by porphyropsin. Possibly an analogous process involving a different pigment, visual violet or iodopsin, is concerned with cone vision in the chick and, maybe, in other animals. According to Wald, the relative amounts of vitamins A and A₂ involved in the visual processes of any species are related with the taxonomy and evolutionary status of the species and so are of fundamental biological importance.

4. Vitamin A and Lesions of Bone, Nerve, Skin, and Teeth

The connection between vitamin A deficiency and the formation of calculi in the bladder and kidney has been appreciated for some ten years, following the publications of van Leersum (334, 335). It has been confirmed by various authors, including McCarrison (336), Wilson and Mookerjee (337) and Gray (338). The view that vitamin A is concerned in calcium metabolism has received unexpected support from the highly significant work of Mellanby and his associates. The development of nerve lesions in advanced avitaminosis A is a well-established observation (339, 340, 341, 342, 343, 344, 345, 346) and that the dental nerves were particularly liable to suffer has been shown by King (347) and King, Lewinsky, and Stewart (348). In more recent publications Mellanby (349) has shown that the involvement of the auditory nerves is accompanied, and possibly preceded, by an astonishing hypertrophy of calcified tissue, involving a degree of distortion of the nerve itself possibly sufficient to account for its degeneration.

Skin lesions are a common clinical manifestation of advanced hypovitaminosis A; the matter has been given particular attention by Mackay (350) and by Goodwin (351).

These various manifestations, as well as the mucosal metaplasia and the effect on dark adaptation discussed above, make it difficult to justify the attachment of any single "descriptive" adjective to vitamin A, whether "anti-infective", proposed by Mellanby, or "anti-keratinizing", as suggested by Harris, Innes, and Griffith (352).

5. Vitamin A Requirements

Daily requirements for any essential nutrients will be placed at different levels according to the nutritional aim in view. The amount just necessary to prevent overt signs of deficiency disease is certainly less than that required to maintain apparently normal health; many authorities believe that this quantity is again less than the amount necessary for "buoyant," "abounding," "positive," or "optimum" health. The work of Sherman (see, for example, A. B. Roher and H. C. Sherman, 353; R. W. Little, A. W. Thomas, and H. C. Sherman, 354) demonstrates how much greater must be the intake of vitamin A if reserve stores are to accumulate in the liver than if normal increase in body-weight is the criterion of adequate vitamin A consumption.

The tendency of nutritionists to state requirements in terms of optimum health was made manifest in general terms by the Hot Springs Conference of 1943. The most recent suggested standards, as given in "Recommended Dietary Allowances" (National Research Council, 355) also show a marked move in that direction, away from the more conservative estimates of some earlier expert bodies. The Council, adopting the recommendations of its Food and Nutrition Board, recommends 5,000 Units per day for adult men and non-pregnant women, 6,000 during pregnancy, 8,000 during lactation, and values from 1,500 to 6,000 for children of varying ages. The recommendation of 6,000 for boys at 16 to 20 years is 1,000 Units greater than that for an adult man of 70 kg., irrespective of his output of physical work.

Information about the requirements of domestic animals has occasionally been published, but the figures are open to some criticism in that the experimental work on which they are based has frequently been marred by inexactness of the kind discussed above in Section VIII. There is, however, an interesting finding of Guilbert, Miller, and Hughes (356) which suggests that the vitamin A requirements for different animal species bear some relation to their body-weights.

In connection with vitamin A requirements, the possibility of hyper-vitaminosis following massive doses needs a reference. A few published results have suggested that high doses of fish liver oil, or of concentrates prepared therefrom, may produce untoward symptoms in experimental animals (*cf.* Chevallier, Cornil, and Chabre, 357); the comments of Eddy and Dalldorf (358) on this matter provide a critical corrective to illegitimate

inference. They write: "The danger of high potency vitamin A preparations does not apparently lie in their content of vitamin A, but in the presence of other factors of a toxic nature, . . . of hypervitaminosis A *per se* we have no evidence today." If this view is correct, there is no necessary danger to be anticipated from continuous and high dosage of vitamin A, provided the preparation used is free from undesirable contaminants. Moore and Wang (362) have, however, recently described a condition of fatal uterine haemorrhage in rats consuming up to 50,000 International Units of crystalline vitamin A acetate daily for 8 to 20 days. The general haemorrhagic condition that results from such vast doses has, they say, "a strong superficial resemblance to that found in scurvy." This is, perhaps, a significant observation when taken in conjunction with the finding of Jonsson, *et al.*, cited on p. 198. The toxicity of bear and seal livers is attributed by Rodahl and Moore (375) to their high vitamin A content.

From what has been written earlier in this review about the stability of vitamin A and carotene, it is to be expected that both would show a certain degree of oxy-thermolability, especially at acid pH ranges, but that ordinary commercial processing of foods, such as canning and pasteurization, and the less vigorous culinary practices, are not likely to bring about serious destruction of vitamins or pro-vitamins. Studies of this matter have not been extensive, but work at the National Institute for Research in Dairying (Reading, England) and the Rowett Institute (Aberdeen, Scotland) confirms this view in connection with the commercial pasteurization of milk (359) and with its sterilization (*cf.* Gillam, Henry, Kon, and White, 360). The danger of losses of vitamin A if it is ingested with rancid fats appears also to have been exaggerated (Lease and Steenbock, 361). Differences in effect of both carotene and vitamin A have been found in experimental animals by Lathbury and Greenwood (363) according to the solvent used, but there is no evidence available as to the existence of corresponding differences in human utilization, except that van Eekelen and Pannevis (364) detected a difference in absorption of carotenoids by human subjects, according as they were given leafy vegetables or a solution of the pigments in vegetable oil. The conclusion of Coward (365), however, was that the rat utilizes, without significant difference, both vitamin A and carotene, whether presented in cod liver oil, in plant tissues, in butter, or as oil solutions of vitamin A concentrates.

Mineral oils, however, as might be expected on physico-chemical grounds, have been found by various authors (*e.g.* Curtis and Ballmer, 366; With, 367) to interfere seriously with intestinal absorption of carotene, but less with that of vitamin A itself.

It seems clear that animals vary greatly in their ability to absorb carotenoids even under the best conditions. De (376) found rats to excrete only

3-5% of ingested vitamin A, but about 42% of ingested carotene. Wald, Carroll, and Sciarra (377) showed that human subjects excrete little or no vitamin A, unless fed huge doses, but excrete about 8% of ingested xanthophyll and 60% of ingested carotene, when any of these substances is fed dissolved in digestible oils. The latter authors consider that these differences account largely for the relative potencies of vitamin A and carotene both in the rat and in man, as well as for the lower relative effectiveness of carotene in man as compared with the rat. There may also be specific differences in the efficiency of conversion of carotene to vitamin A. The consequences of such facts, in assessing vitamin requirements of man or of farm animals, are that the vitamin A value of foods containing carotene must be "written down" to one half or even one third of the nominal number of International Units due to the carotenoids present.

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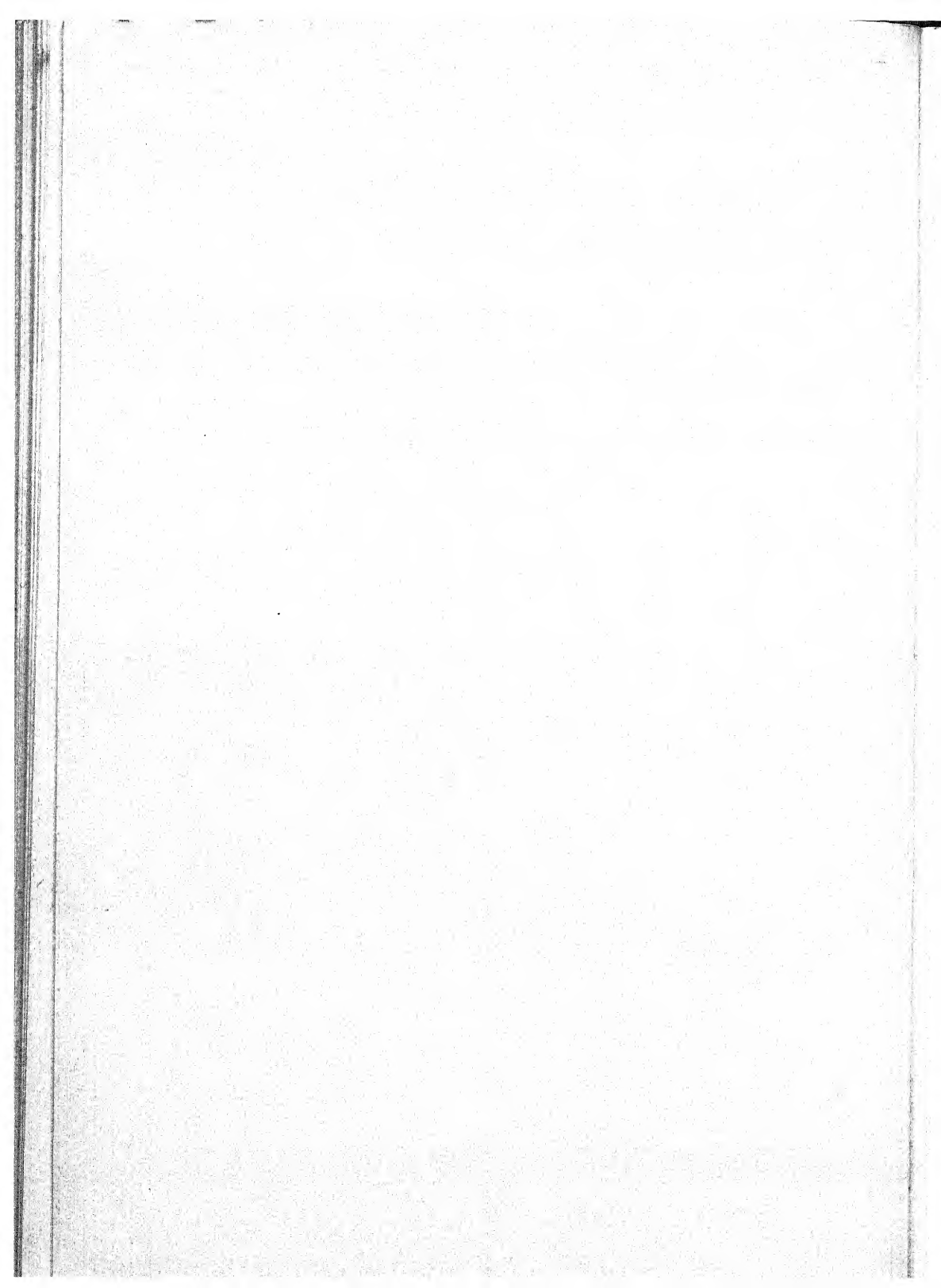
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Para-Aminobenzoic Acid—Experimental and Clinical Studies

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A. Introduction

Para-aminobenzoic acid, abbreviated to "PABA" throughout this article, has been known since Fischer (1) synthesized it in 1863 by reduction of

4-nitro-benzoic acid with ammonium sulfide. The pure substance crystallizes in colorless needles which upon ageing, particularly under the influence of air and light, acquire a yellowish cast. It is freely soluble in alcohol or boiling water. At room temperature, 1 part of PABA may be dissolved in 200 parts of water. Among its incompatibilities are ferric salts and strong oxidizing agents. Although by itself without influence on the sense of touch or pain, practically all efficient local anesthetics are ester-like combinations of an amino alcohol with PABA or with another benzoic acid derivative (2). As will be described, PABA has physiological activities which were intensely investigated since its rôle in pigmentation processes and its sulfonamide antagonism were postulated.

B. Experimental and Clinical Results

I. PIGMENTATION PROCESSES

1. *Sunburn and Suntan*

The darkening of skin resulting from exposure to direct sun rays is a well known phenomenon. The photochemical theory (3) of "sunburn action," *i.e.*, the action of the sun's rays on the skin, may be divided into two parts:

1. A substance is present in the photosensitive layers of the skin, the absorption spectrum of which is identical with or similar to the "sunburn action" spectrum.
2. This substance absorbs the effective Dorno rays, is photochemically altered, and its reaction product causes erythema.

What is the nature of this substance?

Sunburn and suntan of human skin are due to the action of ultra-violet light rays within the Hausser-Vahle (4) field with a maximum effectiveness at 2975 Å. Rothman (5) showed in 1926 that the PABA derivative procaine selectively absorbs the rays causing persistent erythema and pigmentation. He injected a one per cent solution of procaine hydrochloride intradermally and irradiated the site of injection as well as the surrounding skin with ultra-violet light. After the usual latent period, the typical light dermatitis was observed. However, on the second, third, and fourth day the papilla resulting from the injected drug seemed to be covered with skin as normally pigmented and as free from inflammation as the non-irradiated skin; but the tissue surrounding the papilla showed the typical light dermatitis. Two years later, the selective absorption of the Dorno rays was found (6) to be caused by PABA or its derivatives, namely, by compounds with an amino and a carboxyl group in para position on the benzene ring, including PABA with substituted hydrogen atoms of either or both polar groups. It would seem, therefore, that PABA and some of its derivatives may be effective as filters for the erythema producing rays. This conclusion was first reached

by Bird (7) who had studied the ultra-violet absorption of surface anesthetics. He obtained good results with the relatively water-soluble PABA derivative larocaine salicylate. Later Rothman and Rubin (8) prepared a product extremely effective against sunburn by incorporating PABA *per se* in ointment bases.

The absorption of PABA is maximum at 2785 Å (9, 10) which is close to that of proteins (11) principally due to their tryptophan content. At this maximum, PABA's molecular extinction coefficient is three times that of tryptophan, but it is three hundred times greater at 3000 Å, the shapes of the bands being different. These qualitative differences in the maxima of both compounds were demonstrated (8) and the extinction coefficients calculated at different wave lengths in percentages of the maxima, taking the maximum absorption of both compounds equally as one hundred per cent. It thus became evident that the absorption curves of tryptophan and of proteins lie outside of the highest points of the "sunburn action" spectrum which was, however, completely comprised by that of PABA. It is of interest that acetylated PABA has an absorption band that does not include the sunburn rays.

PABA was noticed to be capable of producing erythema when irradiated in saline solution and subsequently injected intracutaneously (8). This reaction was not obtained with proteins, tyrosine or tryptophan similarly irradiated or when non-irradiated PABA was injected. The active principle was postulated to be a yellow oxidation product of PABA; it is probably one of the imines which are known to cause tissue irritation (12). The inflammatory response to irradiated PABA appeared several hours after injection, and this delay is comparable to the latent period in sunburn. One may conclude, therefore, that PABA fulfills the second part of the sunburn action theory. With respect to the first part, it is reasonably certain that PABA is a natural constituent of the skin, since it was found in all body tissues analyzed to date (13).

2. Gray Hair

a) *Terminology.* The graying of hair should be considered among the pigmentation processes of the skin. Loss of hair color is called "achromotrichia" by most biochemists in accordance with the Wisconsin workers (14), although the American Medical Association (15) seemed to prefer the term "achromachia" and Lachat (16) favored the word "canities," which was defined by Oppenheimer and Kugel (17) as "premature graying of hair."

b) *Theory.* Hrdlička (18) theorized that graying is an automatic symptom of metabolisms declining with age and that no drug or chemical can be expected to have more than a temporary stimulating effect on the formation of melanin pigment by the organism. Gibbs (19), however, pointed out

that coloring of the hair is functional in character and not automatically dependent upon some generalized bodily alteration, as evidenced by the seasonal changes in a variety of fur-bearing animals. It should be possible, therefore, to modify the coloration of fibers by pharmacological agents. According to Ballenger (20), graying of hair may be due to a vitamin deficiency, and the American Medical Association (21) published a statement by anonymous competent authorities recognizing the possibility of a systemic influence on the color of human hair: "The old idea that hair is practically a dead tissue, cut off from the metabolic influences of the body, must be forsaken."

c) *Factors.* Biochemists have been wondering for the past years whether there are factors capable of overcoming the decline in the production of melanin, or of enabling the animal organism to start once more the formation of this pigment. Various names have been proposed, such as anti-gray; anti-gray hair; anti-canitic; trichochromogenic (22); anti-achromotrichia, or chromotrichia(1) factor, and many different compounds have been claimed at one time or another to be involved in achromotrichia.

Practically all the workers agree that graying of the fur in the black or piebald rat and feather depigmentation in the chick (23) may be brought about by a deficiency in one or more of the B-vitamins. The work on the relation of loss of color of the fur and the vitamin B complex dates back to 1938, when Lunde and Kringstad (24) in Norway, and Morgan with her collaborators in California (25) published their first papers almost simultaneously. In April 1940, a crystalline material was isolated and claimed (26) to be highly active in the prevention of rat achromotrichia and distinct from all the then known members of the vitamin B complex. Four months later, the curative action of pantothenic acid was observed (27) on nutritional depigmentation of the fur of rats fed diets deficient in this vitamin. Shortly afterwards, a report (28) appeared ascertaining the prophylactic and therapeutic efficacy of pantothenic acid in this type of graying. Subsequently, pantothenic acid was found (29) not to restore pigmentation of the fur completely nor to fully prevent depigmentation. Indeed, at the end of 1940, the inefficacy of pantothenic acid against the graying of fur was claimed (30). Nevertheless, pantothenic acid is definitely one of the factors which prevents nutritional graying in rats and mice, and biotin may have some influence in conjunction with it (31).

Symmetrical achromotrichia was observed (32) to persist in black rats maintained on dried, fresh egg-white containing rations even after therapeutic attempts had been initiated by the feeding of biotin. The gray pattern was the reverse of that occurring in pantothenic acid deficiency. Since the graying of the pelage persisted to some extent even following five

weeks of biotin supplementation, the diet employed was obviously incomplete with respect to chromotrichial factors.

The graying of the fur of the black or piebald rat was compared (33) to the rusting produced in albino rats by omission of choline and pantothenic acid from the diet. Furthermore, rat achromotrichia was shown (34) to result not only from a deficiency of a vitamin B complex factor or factors but also from a dietary lack in iron, copper, and manganese. The somewhat beneficial effect of cystine was demonstrated (35) in the graying of rats brought about by pantothenic-acid-deficient diets. Although the inefficacy of hormones in this type of achromotrichia was reported (36), more graying in male than in female mice was found (37), indicating graying to be possibly dependent upon some hormonal factors. Indeed, partial pigmentation in albino rats was noticed (38) following the subcutaneous implantation of pellets of various hormones, such as androsterone, estrone, α -estradiol, estriol, equilin, stilbestrol, but not testosterone; moreover, rusting in desalivated rats was noted (39) in female animals maintained on a milk diet. A disturbed relationship between the B-vitamins and sex hormones appears to be suggested by the above data as a cause of faulty pigmentation.

Up to 1941, the diets of experimental animals employed in vitamin B complex studies were usually supplemented with no more than the six basic B-vitamins [thiamine (B_1), riboflavin (B_2), pantothenic acid (B_3), choline (B_4), niacin (B_5), pyridoxin (B_6)]. More recent investigations centered around additional factors and PABA was suggested (40) to have chromotrichial activity in the rat reared on a diet adequate with respect to the six basic B-vitamins and containing inositol. In a curative test a bluish discoloration of the skin was noted within the first two to three weeks of PABA supplementation. According to György, *et al.* (41), the appearance of dark colored skin is a first sign of blackening of the fur, due to growth of normally pigmented hair shafts in the epidermis. This observation was subsequently confirmed by Ralli and Graef (42) who noticed that the skin was uniformly pink in black rats on achromotrichial rations whereas blue pigmented areas in band form were present in animals on complete rations. These bands are seen because of the visibility of the concentrated melanin in the hair bulbs through the thin overlying cutis. A diet deficient in the anti-gray hair principle causes cessation of melanin deposition with resulting graying of hair. In the curative experiment, the skin was noticed to develop a bluish color which reached its greatest intensity on the fourteenth to sixteenth day. By the end of the second week, new fur became visible and was either glossy black or brown, depending on the original color of the pelage. In marked contrast, no pigmented bands appeared, and new hair was of a dull gray in rats continued on deficient rations. The pigmented stripes are proof of deposition of melanin, since the "dopa oxidase" reaction

(43) was strongly positive in the active hair bulbs and in the follicles within the bluish-black colored areas, but negative in those within the pink zones of the skin.

The anti-gray hair activity of PABA was confirmed (44) in mice rendered gray by the administration of hydroquinone, the achromotrichial activity of which had been reported earlier (45). Depigmentation in black and black-hooded animals may also be brought about by the oral administration of other "toxic" substances, such as phenylthiocarbamide (46), *o*-aminoazotoluene or 2,2'-azonaphthalene (47). In nutritional mouse achromotrichia, PABA was found (48) to be equally effective as a rice polish extract (49).

Three independent groups of workers (50-52) published soon afterwards that the graying brought about by pantothenic acid deficiency is not alleviated by PABA. The American Medical Association (15) consequently stated editorially that the evidence in behalf of some definite relationship between intake of vitamins and graying of hair was far more convincing for pantothenic acid than for PABA. Martin (53) attempted to bring the apparently contradictory observations into harmony. He confirmed the results of the three laboratories (50-52) which were unable to demonstrate PABA's chromotrichial effect, since he found the six basic B-vitamins adequate for seemingly normal nutrition. However, he showed that these factors were not sufficient when either inositol or PABA was added to the diet. It seems certain that the stimulation of growth of microorganisms by one member of the vitamin B complex causes an increased synthesis of another member. PABA precipitates an inositol deficiency (48) either through stimulation or inhibition of bacterial growth. Indeed, an apparent inhibition of *Proteus vulgaris* and a stimulation of lactic acid forming organisms was noted (53), the overgrowth of the latter being possibly the cause for the seeming inhibition of the former.

In a study of the rôle of sulfonamides and PABA in altering the microflora of the intestine of rats fed a ration containing the six basic B-vitamins and inositol, PABA was found (54) to have marked growth-promoting properties and to be life-sustaining. Sulfanilamide prevented the development of the syndrome heretofore associated with PABA deficiency (53), but it did not entirely replace it. On the other hand, Pfaltz (55) observed no definite deficiency symptoms from lack of PABA in the diet, but noticed that the latter compound was capable of completing or sustaining the favorable prophylactic and curative effects of pantothenic acid in nutritional rat achromotrichia. Dann and her collaborators (56) likewise obtained results indicating that PABA may supplement the influence of pantothenic acid; they postulated an as yet unknown additional factor or group of factors, since liver (57) and yeast feeding resulted in more pronounced curative effects than the various combinations of synthetic B-vitamins. However,

Martin (58) contended that the failure to observe a more definite efficacy of PABA may be due to the fact that its physiological activity is dependent upon its quantity in the diet relative to that of pantothenic acid. Incidentally, the Wisconsin investigators (59) noted that the golden hamster needs in addition to pantothenic acid either PABA, or inositol, or both, since deaths occurred when both these factors were excluded from the ration.

II. DIETARY STUDIES

1. *Sulfanamide Rations*

The more recent PABA investigations made use of sulfanilamide type drugs to inhibit the synthesis of nutritional factors by bacteria. Sulfaguanidine, described by Marshall, *et al.* (60) in 1940 as an effective bacteriostatic agent for intestinal organisms, is now known to cause failure to maintain normal growth in rabbits (61), rats (62), and chickens (63), when fed in large amounts over a long period of time. At least some of the harmful effects can be counteracted with liver extract (64, 65), vitamin K (66), biotin (65, 67), and with PABA (64). The latter substance gave a definite growth response, although none during the first week, when administered orally to rats which had received a diet containing one half per cent of sulfaguanidine for several weeks. This observation seems to indicate that PABA reduces the toxic effects of the sulfonamide on certain bacteria in the intestine; when these bacteria are eliminated from the tract before PABA is administered, it takes at least a week for the bacteria to become re-established.

The growth-promoting activity of PABA was confirmed (68) with rats fed a sulfaguanidine diet supplemented with the six basic B-vitamins, inositol, and biotin. A graying of the fur was noticed which could be cured by "folic acid" concentrate. Thus, the nutritional achromotrichia seems to be dependent upon at least three major factors, namely, pantothenic acid, folic acid, and PABA. The latter appears to play a rôle only in so far as it alters the intestinal flora, thus favorably influencing the bacterial synthesis of folic acid, for which *Escherichia coli* organisms were noted (69) to be mainly responsible. A similar explanation was offered by Wright and Welch (70) who reasoned that the controversial anti-gray hair action of PABA and its growth-promoting effects may be due to a stimulation of the intestinal synthesis of folic acid and perhaps of biotin, and that these latter factors, in turn, improve the utilization of available pantothenic acid. Such a hypothesis suggests that any anti-gray hair activity of PABA may be mediated through folic acid and biotin, and that the effect of the latter substances is secondary to that of pantothenic acid.

Sulfasuccidine is perhaps more suitable than sulfaguanidine for studies

on the relationship of B-vitamins to bacterial flora, because PABA was found (71) not to abolish its inhibiting effect on the growth of animals. Although this result was confirmed (72), the antagonistic action of PABA could nevertheless be demonstrated, since the sulfonamide led to the production of a hypoprothrombinemia which was inhibited, but not completely blocked, by PABA. Subsequent investigations (73) showed that PABA partially counteracts the effect of sulfasuxidine on vitamin syntheses in the intestinal tract. The fundamental and complex relationship of B-vitamins and intestinal bacteria was further emphasized in studies (74) with baby chicks showing that the number of deaths resulting from the administration of sulfaguanidine at a level of one per cent of body weight per bird per day was doubled by PABA given orally with thiamine and riboflavin, and nearly tripled by *E. coli* feeding. Incidentally, an inhibition of a vitamin synthesis, namely vitamin K, by PABA had already been suspected (75) in chicks reared on a heated diet deficient in prothrombinogenic factors. Moreover, the importance of bacterial flora in human deficiency diseases, such as graying of hair resulting from protracted illness associated with gastrointestinal disorders, is generally acknowledged.

2. Chick Nutrition

As mentioned in the preceding chapter, the addition of PABA to a heated diet low in vitamin K advanced the time of occurrence of the hemorrhagic diathesis typical for the hypoprothrombinemia of the baby chick (75). The simple aromatic amine was claimed (40) to be a growth factor for these animals and to prolong their survival times on a heated grain mixture supplemented with the fat-soluble vitamins A, D, and K, with the basic B-vitamins and with inositol. It is to be noted that this diet was deficient in biotin, folic acid, and perhaps other, as yet unidentified, factors. According to Hammond, *et al.* (76), the chick requires PABA for the development of the keel bone when reared on a heated ration deficient in phosphorus. However, the Wisconsin workers (77) were at first unable to demonstrate that the simple aromatic amine is needed by birds maintained on a heated diet. Later, they (78) reported studies on its growth-promoting activity and discussed a possible mechanism of action. Apparently, 5 mg. of PABA per 100 g. of diet had no effect, whereas levels of 7.5 mg. and 15 mg. gave noticeable responses in both growth and feathering formation in chicks receiving purified rations known to be low in "unknown" vitamins and considered adequate in all other respects. Moreover, PABA was observed to stimulate folic acid production and growth of microorganisms in the intestine of chicks. Therefore, PABA is believed not to be a specific growth factor but to act indirectly by stimulating intestinal bacteria which produce unknown factor(s) essential for the chick. For this reason, PABA was not

listed (79) among the first eleven B complex factors, which are the six basic B-vitamins (see p. 219), biotin (B_7), inositol (B_8), folic acid (B_9), a feathering (B_{10}) and an unknown growth substance (B_{11}). It is of interest to note that the explanation offered for the activity of PABA in chick nutrition is the same as the one given for its rôle in rat metabolism (68, 70).

3. Lactation

Almost every one of the better known B-vitamins has at one time or another been suggested for a crucial rôle in normal lactation of the rat. Sure (80) indicated that PABA should be included in the list of essential dietary factors. He found (81) that the lactation efficiency index rose from 5 to 67 per cent when the simple aromatic amine was supplementing the diet. He concluded that PABA or a substance with similar physiological properties is a component of a new dietary essential for lactation to which he gave the same name as the one assigned by the Norwegian investigators (82) to an anti-gray hair principle from liver, namely, " B_x " factor. Climenko and McChesney (83), however, were unable to confirm all of Sure's data. They observed that 15 mg. of PABA per rat per day delayed initiation of lactation; on the other hand, it did slightly decrease the mortality rates of rats newly born to animals receiving the basic B-vitamins and inositol. Subsequently, Sure (84) published additional data confirming the markedly favorable influence of PABA on lactation of albino rats and showing that the pronounced injurious influence of inositol is counteracted by the simple aromatic amine. The deleterious effect of inositol on lactation and fertility was also observed (85) in experiments with rats on sulfaguanidine rations which were adequate when free from inositol but supplemented with PABA.

III. CLINICAL STUDIES

1. *Achromotrichia*

Although the work on B-vitamins in relation to gray hair has started more than five years ago, only a few clinical papers have been published. According to Major (86), the Good Housekeeping Institute has issued a statement that it is possible to change a human being's hair color to normal by the use of calcium pantothenate. However, Spies and Butt (87) reported that Stanbery and Spies found no change in hair pigmentation as a result of pantothenic acid therapy. Vorhaus, *et al.* (88) similarly concluded that pantothenic acid has little value as a human anti-gray hair factor. More recently, Kerlan and Herwick (89) emphasized the lack of value of calcium pantothenate for human achromotrichia. However, a return to natural color of the hair of patients receiving large doses of

vitamin B complex preparations was mentioned in a publication by Sieve (90) who was first to announce that PABA causes a marked darkening of the hair of the human. He noted (91) in cases in which PABA was effective that "the gray hair was characterized by a yellowish cast," which may be an intermediary product in the oxidation of melanin (92). Subsequently, Sieve (93) claimed the beneficial influence of PABA on the achromotrichia of 82 per cent of his 460 patients.

Similar effects were noted by Banay (94). Some 20 inmates of a penal institution received PABA in the form of a 100 mg. tablet three times daily for periods varying from 6 to 8 months as the sole therapy and without any change in daily routine and dietary regimen. A progressive gradual darkening of the hair occurred, starting at the back of the head from the vertex down to the occipital region. The pigmentation was observed to return in geometric designs or islands instead of following a general distribution. The improvement of the hair color was apparently established through the peripheral nervous system and its ramifications (95).

Eller (96) and Eller in collaboration with Diaz (97) noticed definite changes in the color of the hair of a few of their 88 patients given 100 mg. tablets of PABA 3 or 4 times daily over periods ranging from 3 to 5 months. However, they were not certain that PABA was the causative factor in the darkening of the hair, since the increase in pigmentation occurred in only a small number of cases. Friedgood (98) likewise reported a few patients in whom PABA was shown to have chromotrichial activity under special circumstances. Almost simultaneously, DeVilbiss (99) published her observations on the efficacy of PABA in restoring natural color to human gray hair. The longest period of treatment was 7 months, and a total of 16 patients received an average daily dose of two or three 100 mg. PABA tablets. "All patients report visual darkening of the hair. Photographs taken before and 6 months after treatment support patients' statements." More recently, Brandaleone, *et al.* (100) claimed photographs to be useless, since slight changes in distance or lighting can make large differences in the apparent color of the hair. Because a very definite change must occur in order to become evident, clippings of hair samples from a given area were considered valuable in discrediting subjectively favorable results. In two of seven patients, receiving a relatively small daily dose of PABA together with yeast and a comparatively large amount of pantothenic acid, "unequivocal change" in hair color was found. Both cases were men, and both had brown hair. The change in pigmentation tended toward a return to the original color and became noticeable after 2 or 3 months' therapy, thereafter increasing slowly in intensity. Pantothenic acid was ineffective in doses of 100 mg. given daily to 7 patients. The results obtained with PABA, administered to 5 patients in 200 mg. daily doses, were considered negative.

All in all 19 cases were studied by these investigators; the most common change was the appearance of a yellow or greenish cast to the white or graying hair, thus confirming the observations mentioned by others (91, 92). In several patients a greater luster without actual change in pigmentation was thought to be developed. In this connection it is of interest that a focus of infection or endocrine imbalance was stated (93) to interfere with the proper absorption and utilization of PABA and that 100 mg. doses daily with the meals and at bedtime were recommended (101).

The clinical reports discussed in the preceding paragraphs show that pantothenic acid *per se* has no influence in human achromotrichia. PABA, however, is effective, since all the investigators who presented data found its beneficial influence in at least a small number of cases. As in the animal experiments (40), the simple aromatic amine seems to be best administered in conjunction with the vitamin B complex factors. The American Medical Association (102) in a very recent comment on "Vitamins and Human Gray Hair" acknowledges the favorable observations made with PABA by asking whether comparable results will be obtained when younger people with gray or graying hair are studied.

2. Other Conditions

Friedgood (98) reviewed the attributes that Sieve has ascribed to PABA: "it darkens gray hair; it intensifies the normal pigmentation of the nipples and mucous membranes of the mouth, vagina, and anus; it eradicates areas of vitiligo; it induces a return of color and 'changes the hypertrophy' of areas of leukoplakia in the mouth; it causes a decrease or almost complete disappearance of hyperpigmentation of nevi and freckles; it stimulates libido; it re-establishes the menstrual cycle in amenorrhea and increases the amount of flow in oligomenorrhea; it cures female sterility (12 out of 22 cases); it benefits asthmatic patients in certain instances; and it increases the appetite and induces a feeling of well-being."

Although confirmation of all of Sieve's claims is lacking, the return of pigmentation in human gray hair has been described by others (94-100) referred to in the preceding chapter. Furthermore, Costello (103) published a case of vitiligo successfully treated with PABA. DeVilbiss (99) commented on the stimulation of libido. Banay (94, 95) mentioned a marked increase in erotic drive as well as stimulation of appetite. Other physicians stated in private communications that PABA therapy seems to have a favorable action in chronic constipation and appears to contribute to a feeling of well-being. Most clinicians are of the opinion that asthmatics are not benefited by PABA, although some cases were noted to be favorably influenced (48). Domarus (104) found PABA to be beneficial in 4 cases (psychotic episode with psychopathic personality; alcoholic Korsakow

psychosis; chronic alcoholism; and morphine addiction). He believes that PABA therapy should be further investigated in parasympathetico-tonic conditions and as an adjuvant to morphine withdrawal treatment.

IV. TOXICITY

Although PABA was at first believed (105, 106) to pass unchanged through the organism; later experiments demonstrated that it is rapidly acetylated and that the conjugated amine is readily excreted through the kidneys. (107-113). Hensel (114) observed that acetic acid given with PABA stimulated the elimination of the acetylated amine and concluded that acetic acid serves as acetyl donor. However, Erlenmeyer; *et al.* (115) could not detect deuterium in the eliminated conjugated PABA after the administration of deuterium acetic acid. On the other hand, Bernhard (116) found that about nine per cent of the deuterioacetic acid was used in the acetylation of PABA. It seems, therefore, that the major part of the acetic acid employed for detoxication is obtained from metabolic processes. Fishman and Cohn (117) observed approximately the same amount of deuterium to be present in the acetyl groups, when acetylation of PABA and sulfonamides occurred *in vivo* in a medium containing D_2O . The latter did not enter the preformed acetyl group before or during the process of acetylation. If acetic acid is an acetylating agent that does not appreciably exchange its hydrogen with that of the body water (116), it may be concluded to be formed in the body by an irreversible process which introduces the hydrogen of the body water into the molecule. Partly conjugated PABA was found (118) in the blood and urine of toads, but not in frogs and turtles, nor in dogs (116). Sodium acetate either had no effect or decreased the acetylation of PABA in six out of seven rabbits (118a). Apparently, acetylation depends upon the formation and concentration of acetylphosphate. The latter substance may be synthesized from acetoin or diacetyl (118a) or it may be formed directly from pyruvic acid (118b).

PABA diffuses into the cerebrospinal fluid quite as easily as sulfanilamide. It is probably localized within the cell in a manner which makes some of it unavailable for conditioning its rate of diffusion. Therefore, its tissue/plasma distribution ratio (118c) is not necessarily an expression of its concentration ratio across the cell membrane. Reabsorption proceeds as an active tubular process to a considerable extent in the renal tubules. PABA does not participate in a mechanism of active tubular excretion, although in general strongly polar molecules do. Para-aminohippuric acid, PABA's glycine derivative, was observed (118d) to be actively excreted by the renal tubes.

Strauss and collaborators (119) found following ingestion of one to four grams of PABA that maximum blood levels were obtained in one to two

hours and that the concentration in plasma was about three times as great as that in red cells. Conjugation began early and rapidly, and no PABA could be detected in the blood four hours after the administration of two grams. Six hours after the intake of four grams, there was only a small amount of PABA in circulation and complete recovery from urine was obtained in twelve hours. Similar results were reported by Kirch and Bergeim (120) who were able to detect a small but definite amount of non-acetylated PABA in the urine within one hour after ingestion. Even after twenty-eight hours, a measurable quantity of conjugated PABA was found. Zehender (121) determined that PABA is more quickly eliminated than sulfathiazole and that the blood level of the former does not reach as high a maximum as that of the latter. Within twenty-four hours, 75 per cent of one gram of orally administered PABA was noted to be eliminated and only the acetylated compound could be measured in urine and serum.

According to Miller (122), 80 mg. of the sodium salt of PABA was well tolerated but 160 mg. was lethal subcutaneously or peritoneally for mice. Scott and Robbins (123) reported PABA to have a remarkably low toxicity, namely an oral median lethal dose of 2.85 g./kg. for mice, over 6 g./kg. for rats, and 1-3 g./kg. for dogs. The corresponding intravenous values were 4.60 g./kg. for mice and 2.76 g./kg. for rats. In chronic toxicity studies, rats were noticed to tolerate daily doses of 1.4 g./kg. by mouth for about a month without inhibition of growth or pathological changes. These data were confirmed by Richards (124) who emphasized the low acute and chronic toxicity of PABA on the basis of the following results: 4 g./kg. intravenously was the lethal dose for 30 per cent and intraperitoneally for 40 per cent of the rats. No deaths occurred unless at least 6 g./kg. was fed daily for 3 successive days. Young rats injected with doses of 0.2-0.5 g./kg. daily for 21 days had a growth rate slightly better than that of the controls.

Strauss and Finland (125) claimed that they noticed no untoward effects from PABA administered in massive doses to humans. One of the patients received 2 g. at 3-hour intervals for 12 hours and another one ingested a total dose of 62 g. of PABA in less than 2 weeks, namely 29 g. at 2-hour intervals during a period of 52 hours and subsequently 33 g. over a 66-hour period. Other investigators (91, 94, 100) confirmed the low toxicity of PABA.

V. DETOXICATION

PABA, detoxified by acetylation in the organism as described in the preceding chapter, is a detoxicant by itself. Symptoms of hydroquinone poisoning were shown (44) to be overcome by its oral administration. It was found (126) to be highly effective in rats as a detoxicant for lethal

doses of Carbarsone and other pentavalent arsonates important in the treatment of neurosyphilis, and not to inhibit their trypanocidal action. Later, Sandground (127) reiterated PABA's detoxifying action of Carbarsone, "Tryparsamide," arsanilic acid, Acetarson, of pentavalent arsenicals including phenylarsonic acid, and of at least one trivalent arsenical anti-syphilis drug. PABA's protective properties against the toxic effects of antimony compounds employed in tropical diseases and particularly against the pentavalent "Stibosan" were claimed to be spectacular. However, Peters (128) found no reduction in the acutely fatal toxicity of "Mapharsen" in mice by the prior administration of either PABA or methyl-*m*-amino-*p*-hydroxybenzoic acid. It is interesting in this connection to record the lack of activity of PABA in carbon tetrachloride poisoning, against which sulfonamides were observed to protect (129).

György and Tomarelli (130) found that of all the known B-vitamins only PABA has proved to be significantly antioxygenic in the heterogeneous system consisting of corn starch, linoleic acid, and butter yellow. However, the antioxidant inherent in potent sources of the vitamin B complex, such as yeast (131), rice bran, and liver, was stated not to be identical with PABA. In view of the close structural similarity of PABA with other aromatic amines, such as aminophenols, which are commonly employed as antioxidants in the preservation of rubber and oils (132), the authors thought that PABA's antioxygenic property could almost have been predicted.

VI. HORMONES

The possible relationship of PABA to certain hormones was first mentioned in a report (91) describing that 12 out of 22 females, who had been sterile for a *minimum* of 5 years, conceived in normal marital relations after having ingested a 100 mg. PABA tablet daily with each of 3 meals and a 4th tablet at bedtime during periods varying between 3 and 7 months. Furthermore, PABA's inhibition of thyroid hormone production has been reported (133), and it has been stated (134) that combined PABA and stilbestrol therapy may result in anovulatory menses. A basis for the clinical contention was offered by experiments (135) showing PABA to be capable of inhibiting the enzymatic destruction of the synthetic estrogen. Since large amounts of administered estrogenic material are not excreted in appreciable quantities, it seems that they are rapidly destroyed or altered beyond simple conjugation within the animal organism [liver (136)], probably by enzymes (137, 138). The possibility of enzymatic inactivation was demonstrated pharmacologically (139) and by the Warburg technic (140). With the latter method, stilbestrol was observed (141) to be oxidized by mushroom tyrosinase with greater ease than either estrone,

α -estradiol, or estriol. However, the rate of oxidation was greatly retarded by PABA. This result was confirmed with the former method; namely, the activity of the synthetic estrogen was reduced to $\frac{1}{3}$ of its original value by incubation in the presence of tyrosinase; when PABA was added before incubation, $\frac{5}{8}$ of the activity remained.

PABA is capable of inhibiting the destruction of other hormones, such as adrenaline (141). As determined in the Warburg apparatus, various benzoates have a great influence on the oxidation of adrenaline catalyzed by mushroom tyrosinase. Benzoic acid inhibited even more than did PABA, the isomers of which were almost equally active. Sulfanilamide had a small, nevertheless definite accelerating effect. The protection afforded to the hormone by the various benzoates against enzymatic destruction has also been ascertained pharmacologically (142). These data would seem to offer a basis for an explanation of some of the clinical results obtained with PABA in cases of asthma (48).

In view of the fact that a diabetic patient went into shock as a result of combined PABA and insulin therapy (91), it is of interest that PABA appears to potentiate insulin (143). Insulin was injected subcutaneously into mice in a dose ($\frac{1}{3}$ unit per 18 g. of body weight) which is known not to reduce the blood sugar to convulsive levels. When PABA was given either before, simultaneously, or after the insulin, convulsions typical of insulin shock were produced. At the same level of 1 g. per kg. of body weight, the simple aromatic amine effected a mild but definite hyperglycemia in rats. It did not alter the speed, degree, or duration of the hypoglycemia produced in rabbits by insulin. As determined by the method of Grattan and Jensen (144), it was noted to deplete the liver of glycogen. Since mice treated with a cortical extract and PABA had an average liver glycogen content of about 500 mg. per cent as contrasted with approximately 1700 mg. per cent found in the livers of animals which had received solely the cortical extract, it may be concluded that the rate at which the organism is required to yield its stores of liver glycogen is augmented by the simple aromatic amine. Conditions favorable for insulin potentiation by PABA have not been found in clinical trials.

The above data were discussed and extended by Martin (145) who considered the following facts:

- 1) PABA acts in the pseudo-potentiation of insulin (143, 145);
- 2) it produces a mild hyperglycemia in doses of 1 and 2 g. per kg. in dogs (143);
- 3) it depletes the glycogen reserves of the liver (143);
- 4) it causes a mild rise in blood pressure in the anesthetized cat, reversed by 2-(1)-piperidylmethyl-1,4-benzodioxan (146);
- 5) it is known to inhibit various enzyme systems (142);

- 6) it produces hypertrophied thyroids with hyperthyroidism (145, 147, 148); and
 - 7) it protects against the action of thyroxine in the intact animal (145).
- Martin suggested that these actions of PABA may be motivated via the pituitary and production of thyrotrophic hormone, as already concluded by Astwood, *et al.* (148) and Mackenzie, *et al.* (147). Therefore, PABA may be effective clinically in counteracting hypothyroidism and may be contraindicated in cases of hyperthyroidism. Indeed, the action of a number of agents, including thioureas, sulfanilamide and PABA in producing hypertrophied thyroids (147-151) has been demonstrated. These various substances may be considered to be inhibitors of tissue metabolism, as first suggested by Cutting and Kuzell (152). The pseudo-potentialization of insulin by these compounds as well as by others, such as *e.g.* saccharin (153), can best be explained at present on the basis of the above consideration.

VII. ENZYMES

1. *Rôle in Systems*

Fildes (154) postulated that PABA is an essential metabolite associated with one or more of the enzymatic processes involved in bacterial growth. By definition, an "essential metabolite" is a substance that takes an essential part in a chain of syntheses necessary for bacterial growth, in contradistinction to a "growth factor" which must be supplied in the nutrients as an essential metabolite which the cell cannot synthesize. Fildes emphasized the close structural relationship between PABA and sulfonamides and suggested the latter may act by blocking the enzyme system(s) with which the former is involved and on which bacteria depend for normal growth and development. Proof that PABA is truly an "essential metabolite" was given by Landy, *et al.* (155) who found representative cultures of many bacterial genera to elaborate PABA in readily measurable amounts.

With respect to the second part of Fildes' hypothesis, it has been shown (156) experimentally that the inhibition produced by sulfanilamide type drugs is competitive (157) with respect to PABA. Employing PABA and sulfanilamide simultaneously in equimolecular quantities in *p*-cresol-tyrosinase systems, an increased rate of oxidation was found which was only slightly less than that obtained when PABA alone was used. PABA has apparently a greater affinity for the enzyme than have sulfanilamide type drugs. The sulfonamide inhibition as influenced by PABA was mathematically analyzed (158) and the linear relation between the two substances was confirmed (159), thus indicating competitive inhibitions of an essential enzyme reaction by a substance chemically related to the substrate.

It is now generally accepted that the efficacy of sulfanilamide type

drugs as bacteriostatic agents is due to their structural similarity to the essential metabolite PABA; it is this similarity that enables them to displace the latter from its enzyme surface. Sulfonamides were noticed (160) to have an effect on the electrokinetic mobility of the organisms resembling that of PABA, indicating that they behave like the latter at the bacterial surface. PABA may also be displaced by substances devoid of sulfo groups provided they are structurally related to it, such as *p*-aminobenzamide, Atoxyl (161), and *p*-nitrobenzoic acid (162). There can be little doubt that PABA antagonizes the action of penicillic acid by means of a chemical reaction between the two acids and not by way of a competition for a bacterial enzyme system (163). It is of interest that PABA has been reported (164) to potentiate penicillin. With *Bacillus subtilis*, 1,200 units per ml. of penicillin active in 1:100 became active in 1:6,000, when 1:2,500-1:10,000 PABA was added. With *Staphylococcus aureus*, PABA in concentrations of 1:2,500-1:50,000 increased the penicillin activity from 1:40,000 to 1:75,000-1:1,000,000. The synergistic action of the two compounds was also observed *in vivo* with mice inoculated with staphylococci or streptococci.

Intensity of luminescence of *Vibrio phosphorescens* was noted (165) to be inhibited at temperatures varying from 15 to 35°C. by 0.02 M PABA, more so by 0.03 M sulfanilamide and still further by both of these substances. PABA at high concentrations was observed (166) to inhibit growth and luminescence and to have anti-sulfonamide effect only in growing cultures and not in mature suspensions of luminous bacteria, nor in the purified luciferin-luciferase system of *Cypridina* (167). Since urethane, sulfanilamide, and PABA were noticed to stimulate growth and luminescence in low, while inhibiting in high concentrations, Johnson (168) suggested their fundamental mechanism of action in bacterial metabolism to be in the nature of narcotics. However, evidence is available showing that this interpretation is not justified (169). Nevertheless, barbiturates, chloral hydrate, sulfanilamide, and PABA were claimed (170) to belong to a group of narcotic drugs which decrease bacterial luminescence in a manner that is not reversible by pressure, indicating a decrease in enzymatic activity by an adsorptive process (171). It is to be noted that the inhibition of urethane is reversible and that Johnson's explanation for the mode of sulfonamide action is based largely upon experiments in which the test substances showed enzyme inhibiting rather than stimulating activity. Absence of parallelism between the anti-sulfonamide action of PABA and urethane was demonstrated (172), and the conclusion was reached that the mechanisms prevalent in the luciferase systems are not necessarily those involved in the anti-bacterial action of sulfonamides.

In mushroom tyrosinase systems containing *l*-dopa, *l*-tyrosine hydro-

quinone-catechol, adrenaline, or stilbestrol, urethane was likewise devoid of activity (141, 173). PABA, however, retarded the oxidation of these substrates and caused activation of the rate of aerobic oxidation of phenol, *p*-cresol, and xlenol as catalyzed by enzyme preparations with a high activity ratio of hydroquinone-catecholase to *p*-cresolase (174). Moreover, it was noted to inhibit phenol and *p*-cresol oxidation and to accelerate that of xlenol when the reaction was catalyzed by a high *p*-cresolase type tyrosinase, indicating mushroom preparations to be composed of at least two enzymes, namely, phenolase and *p*-cresolase, the former being activated and the latter being retarded by PABA.

The above data would seem to focus attention to the process of pigmentation which is considered to be enzymatic in character. The classical theory of Raper (175) sets forth that tyrosine is oxidized by tyrosinase to melanin by way of its catechol derivative dopa, the side chain of which condenses with a benzene ring to form an indole. Indole nuclei agglomerate to water-insoluble melanin granules. According to Bloch (176), dopa is the immediate precursor of melanin in mammalian skin and his "dopa reaction" is a specific enzymatic process. In a study of this reaction, PABA was found (177) to modify the formation of melanin; this observation was recently confirmed (47). Lipmann (178), who noted peroxidase from horseradish to catalyze the oxidation of the simple aromatic amine by hydrogen peroxide, also suggested the possible rôle of PABA in pigmentation processes, thus laying a basis for an explanation of a chromotrichial activity.

A chemical model of the bacterial action in which it has been postulated that PABA and sulfonamides compete for a compound not yet specified, was offered by Kohn (179). When rat, rabbit, or guinea pig brain brei was suspended in Ringer phosphate solution and incubated at 38° C. preferably in an acidic medium, a substance "B" was produced which reacted with PABA, sulfonamide, and other substituted aryl amines to form a pigment. Production of "B" involved an enzymatic process and was coupled with respiration, suggesting that the toxicity of sulfanilamide type drugs may be explained in part by their interference with the metabolism of "B".

PABA has been mentioned in still other enzyme systems. It was found (180) to operate in citrated plasma like sulfanilamide; namely, it accelerated the clotting action of staphylocoagulase, an enzyme (181) contained in sterile broth filtrate, and reversed the coagulation inhibitory action of sulfadiazine. Recent studies showed (182) that local anesthetics such as procaine are hydrolyzed to PABA by an esterase inherent in blood. Further investigations (183) demonstrated that human blood plasma and serum can hydrolyze procaine. The split product was isolated and identified as PABA. The hydrolyzing agent was named "procaine esterase" and found in blood, particularly in the serum, and also in liver.

2. *Rôle as Co-enzyme*

As has been emphasized (184), it is important for the understanding of the action of the sulfanilamide type drugs to find the enzyme system(s) in which they have a point of attack common with PABA. There is apparently no relationship between PABA and the co-enzymes with inherent thiamine or niacin amide groups (159). PABA was suggested (174) to be an activator of phenolase. It affected mushroom tyrosinase preparations characterized by high Adam and Nelson catecholase (185) and low Parkinson and Nelson cresolase (186) potencies, indicative of predominating Gregg and Nelson phenolase (187) activity. The latter was activated by PABA to such an extent that it became capable of oxidizing *p*-cresol as well as xylenol and phenol at a great rate and irrespective of the *p*-cresolase content of the enzyme preparations.

Objection to PABA as a natural activator or co-enzyme of what has been called phenolase, rests on the fact that a large amount of PABA was necessary to bring about activation under the experimental conditions employed. However, the simple aromatic amine seems to occur in nature in free and bound forms (188). The latter may have greater affinities for the enzyme than the former and hence may not require large concentrations to cause acceleration. Indeed, extracts comparable to PABA with respect to activation of phenol oxidation (189) were obtained from yeast and dried mushrooms, the PABA content of which is known (188, 190).

VIII. MICROORGANISMS

1. *Sulfonamide Antagonism*

The finding of the anti-sulfonamide action of PABA may be credited to Woods (191) who demonstrated that a factor inherent in yeast and with chemical properties similar to PABA prevents the bacteriostatic action of sulfanilamide and sulfapyridine. This observation has since been extended *in vitro* (119, 192-194) and *in vivo* (195-197) to all sulfanilamide type drugs in all species and media. Green in collaboration with Bielshowsky (198) conceded that the antagonistic action of his "P" factor (193) was due to contamination with PABA, after the latter had been isolated as a benzoyl derivative from yeast (200).

Only the local anesthetics derived from PABA exhibit a blocking action against the *Bacillus coli* bacteriostatic property of sulfonamides (201); this observation was confirmed with pneumococci (202). Nevertheless, PABA substitution may result in compounds inactive or with varying degrees of anti-sulfonamide action (203).

According to Strauss, *et al.* (119), the greater the inhibitory potency of a sulfanilamide type drug upon bacterial growth, the less antagonizing effect has PABA. Since this result was confirmed (204) with *Escherichia coli* and

with *Mycobacterium tuberculosis*, the non-specificity of the bacteriostatic effect of the drugs was demonstrated. This action is directly proportional to the ability of the sulfonamide to counteract the anti-bacteriostatic potency of PABA (205). The antagonism between the former and the latter was observed (206) to be independent of the number of bacteria, but instead to be related principally to critical concentrations of these compounds. The simple aromatic amine counteracts also the activity of 4,4'-diamino-benzil which was claimed (207) to be as typical an antagonist to it as are the sulfonamides.

PABA was found to exert a marked anti-promin effect upon the growth of tubercle bacilli (208) and this action is similar to its anti-sulfonamide effect. Furthermore, it was noted to inhibit the malariacidal action of sulfanilamide type drugs (209), but failed to counteract quinine and atabrin (210), suggesting that the latter compounds affect plasmodia through an entirely different mechanism than the sulfanilamide type drugs. The latter can reduce inorganic catalytic actions like other antiseptics and poisons, but PABA cannot inhibit their anti-catalytic effects (211).

In view of the established rôle of PABA as antagonist, its use was advocated (212, 213) in bactericidal tests to inhibit sulfonamide action. Its addition to all routine culture media was recommended (214), because it will allow rapid growth in cultures from patients under treatment with a sulfanilamide type drug when viable organisms are present.

PABA was found to neutralize also the sulfonamide inhibition of the growth of yeast (215), at least partially that of tomato roots (216), and entirely that of fungi, such as *Trichophyton purpureum* (217). The fact that relatively small quantities were found to be effective in the latter case seems to indicate a preference on the part of the fungus for PABA.

2. Clinical Incompatibility

It has been postulated (218) that PABA may impede the bacteriostatic action of the sulfonamides in the human. Even local anesthetics derived from the simple aromatic amine were thought (182) to be contraindicated, when a sulfonamide is being used in a case of severe infections or massive damage to tissues. The question whether PABA and sulfanilamide type compounds are incompatible clinically can now be answered.

Experimental data have shown (219) that PABA can overcome the antagonistic effect of sulfonamides on the growth of *Escherichia coli* only when the temperature is not above about 37°C. At higher temperatures (37–46°C.), it was noticed to inhibit growth and to have no marked anti-sulfonamide properties. Analogous results were obtained (220) with *Streptococcus pyogenes*, since sulfathiazole, PABA, as well as urea were shown to have an effect on bacterial growth rate which increased with

increasing temperatures. In fact, PABA was found to have sulfonamide-like action at higher temperatures. Moreover, the protective action of sulfadiazine against meningococcus was observed (221) to be inhibited in mice by PABA only when the latter was given repeatedly in such a manner that a considerable concentration of it was maintained in the blood for a prolonged period of time. Serum from patients treated with sulfonamides protected mice against multiple lethal doses of meningococci, and PABA added to such a serum had no inhibitory influence.

PABA was reported (222) to have only a partial effect in suppressing the action of succinylsulfathiazole which affects primarily vitamin synthesizers, i.e. coliform organisms, and leaves vitamin requirers, i.e. lactobacilli, streptococci, and anaerobic bacteria, more or less uninfluenced. Furthermore, it was observed (223) with *Escherichia coli* that sulfanilamide type compounds together with urea are bacteriostatically effective even in the presence of PABA in amounts that inhibited sulfonamide bacteriostasis in the absence of urea. The latter compound seems to potentiate the action of sulfanilamide type drugs (224, 225) in an as yet unknown manner. It is conceivable that a "dynamist" (226), such as urea, may act against an antagonist, such as PABA. However, more recent studies (219, 220) indicated that urea has no synergistic action with sulfanilamide type compounds but reduces the bacterial growth rate sharply at temperatures exceeding 37°C. Indeed, concentrations of urea which are not bacteriostatic were found (227) not to counteract sulfonamide inhibitors, such as PABA, nor to potentiate the bacteriostatic action of the sulfanilamide type compounds against resistant organisms.

It has been admitted (228) that large doses of procaine have no lasting effect in humans and in mice. Nevertheless, the use of drugs not allied to PABA was advocated in cases in which a massive local anesthetic is required and sulfonamide therapy is contemplated. A 20 ml. dose of a 3 per cent procaine solution is believed to prove harmless to a patient whose sulfonamide concentration is 3 mg. per 100 ml. of blood.

In view of the results described in the preceding paragraphs, and particularly on the basis of the fact that the simple aromatic amine is more rapidly excreted from the human body than the sulfanilamide type drugs (119, 229), the conclusion seems to be justified that PABA therapy is not likely to interfere with subsequent sulfonamide treatment.

3. Bactericidal and Fungistatic Properties

Although it has been stated (214) that PABA is not inhibitory to any of the common pathogenic organisms, it might be expected to have antibacterial action, since it is a derivative of aniline. Indeed, concentrations of 0.3 per cent of PABA were observed to be effective against *Pseudomonas*

mors-prunorum (163); it was noticed to inhibit the growth of *Proteus vulgaris* (53) and of *Bacillus cqli communis* (119), and to have slight anti-malarial activity (209). Moreover, small amounts of PABA in combination with a sulfonamide often appeared to inhibit bacterial growth more than did the sulfanilamide type drug alone (230).

PABA's effectiveness against fungi was also investigated. Hoffman, *et al.* (231) found that the fungistatic properties of benzoic acid were decreased by the introduction of a hydroxyl group in ortho and entirely eliminated by substitution in either meta or para position. The hypothesis was advanced that a more powerful fungistat results when the polar groups are concentrated in one area of the molecule rather than spaced around the non-polar nucleus. A condition of resonance may mask the polar nature of amino groups in ortho and para positions, since *m*-aminobenzoic acid showed the non-inhibiting action, whereas the ortho (anthranilic acid) and para (PABA) isomers were found to have greater fungistatic properties than expected (232). Therefore, biological activity of a molecule appears to be related to its structural ability to exist as a resonance hybrid, a fact which may have a bearing on the therapeutic efficacy of sulfanilamide type drugs and the inactivity of their meta and ortho isomers.

4. Physicochemical Data

The ultra-violet absorption curves of various benzene derivatives were determined in acidic, basic and in sodium chloride solutions (233). The general rule was developed that the spectrum of an unsubstituted or alkyl substituted aromatic amine will revert in acid to that of the corresponding substance in which the amino group is replaced by hydrogen or an alkyl group. PABA and sulfanilamide were found to have a higher extinction coefficient in basic than in sodium chloride solutions, indicating that the main resonance form makes a greater contribution in the ion than in the undissociated molecule. PABA and sulfonamides have similar resonating structures with a separation of charge (234) and therapeutic potency may be associated only with forms which have such a charge. The meta isomers do not exist in forms of this type and the ortho isomers may be rendered inactive by way of hydrogen bond formation or through difference of spatial configuration. Since the bacteriostatic action of a sulfanilamide type drug is due to a competition with PABA, it follows that the more closely the spatial configuration of the former resembles that of the latter, the greater is the blocking, *i.e.*, bacteriostatic effect of the former. The molecular ratio of the antagonistic pair, PABA and sulfanilamide (119, 157, 158, 191, 229), is merely a measure of their respective affinity for the unknown receptor site in the organism. These ratios were found (194) to be sensitive and reproducible criteria for the effectiveness of sulfonamides. In a wide

variety of organisms, the receptor site in the bacterial cell is the same, the difference in effectiveness between various sulfanilamide type drugs is a characteristic of each individual drug irrespective of the microorganism involved, and the association of the drug with the organism is a function of the aromatic amino group (158, 160).

PABA has not only a steric similarity to sulfanilamide, but shares also its characteristic attribute to form salts. Both compounds are bases of about equal strength, but the latter is roughly a million times weaker as an acid than the former (235). PABA is characterized by an acidic carboxyl and a basic aromatic amino group in para position to it. Since many of the sulfanilamide type drugs contain the latter and an acidic sulfonamide group, Bell and Roblin (236) made a comparative study of the physicochemical properties of these radicals. The ionization constant (K_b) for PABA's amino group was found to be about 2.6×10^{-12} which is in good agreement with previously reported data (237, 238). The range of the basic strength of this radical of practically all sulfonamides was noted to be small ($0.5\text{--}2.3 \times 10^{-12}$). Therefore, no relation between these constants and biological potency is apparent.

The acid dissociation constants (K_a) of sulfanilamide type drugs were found (236) to vary over a wide range ($<10^{-11}$ to 10^{-8}); the K_a for PABA is 2.1×10^{-5} , its pK_a being 4.68 which is in close agreement with 4.8 reported earlier (239). The carboxyl group of PABA was demonstrated to be better than 99 per cent ionized at pH 7, and the carboxyl ion may increase the basicity of the para amino radical threefold over the value obtained with un-ionized carboxyl in an acidic medium. On the basis of these data, a theory was presented (236) predicting the relative activity of the ionic and molecular forms of the sulfanilamide type drugs.

The anti-bacterial efficiency of a sulfonamide, as measured by its ability to overcome PABA, was observed (240) to be dependent upon the pH of the medium. The former competes on a more favorable basis with the latter at a high than at a low pH where acidic dissociation is relatively small for the weaker acid. Woods (191) had postulated competition for a bacterial enzyme between one mol of PABA and 5–25,000 mols of sulfanilamide. Fox and Rose (241) reported that in excess of 600 times more PABA is needed to block sulfathiazole or sulfadiazine than to block sulfanilamide. However, when only the concentration of ionized drug is considered, all the sulfonamides seem to react in roughly similar amounts with approximately equivalent quantities of PABA. It is apparent that bacteriostatic potency of sulfonamides is influenced by their degree of ionization (236, 241–244) and is a direct function of their protein-combining capacity (245, 246).

The evidence that biological activity of sulfanilamide type compounds

is found in the forms with a separation of charge, is not contradictory with that showing it to be a function of the acid dissociation constant or the negativity of the sulfonyl group (247). The more negative the latter group, the greater is the positive charge on the *p*-amino nitrogen; the form with the separation of charge becomes a more important contributory factor; the amino radical enters into a more coplanar configuration with respect to the aromatic nucleus; and the amino group of PABA is nearly coplanar with the ring. This coplanarity may be a necessary condition for reactivity with enzyme systems, thus explaining the observation that association of the drug with the organism is a function of the aromatic amino group (158, 160). Indeed, when the law of mass action was applied (248) to a system of a sulfonamide and an enzyme in a buffer solution with a pH near 7, it was possible to predict the existence and acid dissociation constant of a drug of maximum potency, to correlate the effectiveness of basic as well as acid sulfanilamide type compounds with their ionization constants, and to account quantitatively for the inhibitory effect of PABA.

The clinical importance of the studies on basicity and drug action has been emphasized editorially (249) and the results obtained were applied to the problem of the local application of soluble sulfanilamide type drugs in war wounds and burns. Since sulfathiazole and sulfadiazine are far more extensively ionized than sulfanilamide (241), infected wounds, the pH of which was observed to range from 5.5 to 6.5, were found (250) to require from 10 to 100 times more of the latter substance for bacteriostasis and only 1 to 4 times more of the former two compounds than less acidic tissues. The solubility of these drugs is also reduced in acidic exudates. Therefore, their sodium salts were recommended for local use in order to obtain prompt control of infection without tissue irritation.

Emphasis has been placed (251) on the absolute necessity of local cleanliness and careful débridement in the external use of sulfonamides. Only under these conditions will the physician be able to prevent formation of the inhibiting PABA and "allied peptone products" derived from broken down pus cells, exudate and bacteria.

5. Growth-promoting Activity

PABA does not stimulate the growth of *Streptococcus hemolyticus* (191), *Streptococcus viridans* (162), *Staphylococcus aureus* (230), or *Pneumococcus* (119), and has not yet been found (252) essential for the normal growth of pathogenic bacteria. However, the lactobacillus growth factor of Möller (253), named "Vitamin H" and observed to antagonize sulfanilamide type drugs (254), was isolated from a "Vitamin H" (biotin) concentrate from yeast and identified as PABA (255). The BY growth factor for

Clostridium acetobutylicum of Oxford, et al. (256) was found to be identical with PABA (257, 258). The simple aromatic amine is needed for growth by 7 strains of *Cl. acetobutylicum*, *Cl. butylicum* No. 28, and by *Cl. felsineum* (Carbone) No. 41 (259); the ethyl ester of PABA was observed to have less than 0.1 per cent activity, although others (260) had found equal potency for the two compounds. It has been stated that PABA in a dilution of 10^{-12} will, in the presence of biotin, stimulate the growth of *Cl. acetobutylicum* (261). That PABA is a true growth factor was confirmed with lactic acid bacteria (262-265), *Cornebacterium diphtheriae* (264), *Acetobacter suboxydans* (266), *Neurospora* mutant (267), *Lactobacillus arabinosus* (263,

TABLE I
PABA in Plant Material

Material	Total PABA	Unbound PABA
	p.p.m.*	p.p.m.*
Alfalfa (Meal)		2 ³
Asparagus (Dried Juice)	2 ¹	1.2 ¹
Cabbage (Dried)	14 ¹	10 ¹
Carrots (Dried)	0.43 ¹	0.18 ¹
Concanavalin ⁶	22 ⁵	0.25 ⁵
Mushrooms	1.3 ²	
Potatoes { (Irish)	0.4 ²	0.3 ²
{ (Sweet)	0.12 ²	0.11 ²
Spinach	0.6 ²	0.12 ²
Tomato (Roots)	+ ⁴	

¹ Data from Reference 263.

* p.p.m. = parts per million.

² Data from Reference 189.

³ Data from Reference 13.

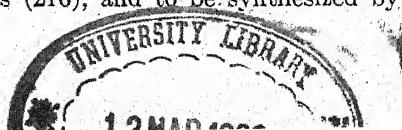
⁴ Data from Reference 216.

⁵ Data from Reference 284.

⁶ Jackbean Protein.

268) and *Clostridium thermosaccharolyticum* (268a). It is of interest to note that the effectiveness of PABA as a growth factor for the *Neurospora crassa* mutant of Tatum and Beadle (267) decreases with the increase in pH of the nutrient solution, indicating activity for the molecular and not the ionic form of the simple aromatic amine (268b).

Stimulation of the growth of *Lupinus albus* seedlings by 1 part per million of PABA has been obtained (269), but stronger concentrations were observed to inhibit though not as much as the PABA isomers. PABA or a substance having similar physiological activity appears to be normally contained in tomato roots (216), and to be synthesized by yeast (215).



IX. DETERMINATION

1. Qualitative Methods

Marshall (270) found that diazotized PABA can be coupled in acid solution with dimethyl- α -naphthylamine or preferably with Bratton and Marshall's (271) *N*-(1-naphthyl) ethylenediamine dihydrochloride. A red pigment is thus obtained which is also formed with diazotized aniline, the PABA isomers, sulfonamides, and related compounds. Nevertheless, the test is specific provided the different reaction rates of the various diazotized substances are taken into account. Another color reaction based upon the formation of a Schiff base was proposed by Tauber and Lauffer (272), who observed that PABA forms a deep yellow color in acetic acid medium with *p*-dimethylaminobenzaldehyde. The test is applicable also to the isomers of PABA, aniline and derivatives, such as *p*-toluidine, but not to aliphatic amino acids nor to aromatic derivatives such as tyrosine or phenylalanine.

2. Quantitative Methods

a) Chemical Analyses. Bratton and Marshall's sulfonamide method (271) has been employed for the quantitative determination of PABA in yeast (188) and blood (273), and full directions were given for the analysis of PABA side by side with procaine (274). Using vitamin B₁ as a reagent, a quantitative method was developed (120) based upon an isoamyl alcohol soluble pink to red compound obtained by reacting PABA with diazotized thiamine.

The use of *p*-dimethylaminobenzaldehyde has been advocated (275, 276) for the determination of sulfanilamide type drugs. This compound was employed (277) in test paper for the estimation of sulfonamides in laked blood. The paper was recently modified by La Rosa (278) for the direct analysis of free sulfonamides and other primary amines, including PABA, in serum. The color developed varies from a pale violet for normal serum to a bright yellow for serum containing the test substances, and may be compared with color standards.

An estimation procedure which makes use of tyrosinase may also be employed (279). When this enzyme catalyzes the aerobic oxidation of catechol, a transient pale yellow-green color is observed. If the medium contains PABA, this color is not noticed, but a red compound is formed which is stable at pH 5. The reaction is sensitive for the colorimetric determination of PABA in amounts of as low as 5–10 γ . The red color produced was found to be dependent upon the amount of PABA, when 10 γ of catechol was added to the tyrosinase solution. The same color is developed, when the system tyrosinase-oxygen is replaced by ceric sulfate, which is easier to obtain and less expensive than the enzyme.

b) *Microbiological Assays.* A semi-quantitative microbiological assay was devised by Rubbo, *et al.* (260) using *Clostridium acetobutylicum*. *Acetobacter suboxydans* was employed by Landy and Dicken (13) who measured in a photoelectric colorimeter the turbidity resulting from the growth of the bacteria. The test is highly specific, since the PABA isomers and other related substances were found to have no biological activity. Because a relationship between purines and PABA exists for growth as well as for reversal of sulfonamide bacteriostasis, the incorporation of adenine, guanine, and xanthine into the basal medium used for PABA

TABLE II
PABA in Animal Tissues

Material		Total PABA	Unbound PABA
		<i>p.p.m.</i>	<i>p.p.m.</i>
Blood	(Human)		0.035 ¹ , 0.015–0.06 ⁵
	(Ox)		0.004 ¹
	(Rat)	0.27 ²	0.06 ²
Urine	(Human)	0.4 ¹ , 0.5 ²	0.02 ¹ , 0.02 ² , 0.015 ³
Spinal Fluid	(Human)		Traces ⁴
Feces	(Dried) (Human)		4–12 ⁴
Liver	(Beef)	2.5 ²	0.2 ² , 0.2 ³
	(Extract, Lilly)		5.0 ³
Meat	(Extract)		1.3 ³
Pork		0.8 ²	0.3 ²
Muscle	(Beef)	0.6 ²	0.3 ²
	(Rat)	1.7 ²	0.15 ²
Brain	(Rat)	0.7 ²	0.14 ²
Heart	(Rat)	1.35 ²	0.15 ²
Kidney	(Rat)	1.8 ²	0.13 ²

¹ Data from Reference 263.

² Data from Reference 190.

³ Data from Reference 13.

⁴ M. Landy, personal communication of July 30, 1943.

⁵ Data from Reference 283.

assays was recommended (280); purines, although not essential for growth, act as growth accessories.

Lactobacillus arabinosus was the test organism in the microbiological assay of Lewis (263), and Mitchell, *et al.* (190) made use of *Neurospora crassa* (mutant). Strong acid or alkaline hydrolysis was found essential (281) to obtain the correct PABA content of natural substances, since it occurs in free and bound forms (188).

Mirick (282) proposed an organism of the *Pseudomonaceae* family which produces enzymes that readily oxidize PABA when grown in the presence of the latter. For this bacillus, PABA is neither a growth factor nor is it

released into the medium in which the bacilli are cultured, but it is completely destroyed as determined by the diazo reaction. The specific adaptive enzymes of this soil bacillus can be used for the identification of as little as 10 γ of PABA. The limitation of the method is primarily a matter of sensitivity of the diazo reaction which allows the measurement of not less than one part of PABA per million. Nevertheless, Mirick recommended this microbiological identification because of the great specificity which characterizes adaptive bacterial enzymes in general and the ones of the proposed bacillus in particular.

TABLE III
PABA in Dairy Products

Product		Total PABA	Unbound PABA
		<i>p.p.m.</i>	<i>p.p.m.</i>
Milk	(Fresh)	0.1 ²	0.08 ² , 0.15 ²
	(Skimmed)		0.004 ¹
	(Whole)	0.4 ²	0.07 ¹
	(Dried)	0.8 ¹	0.2 ¹
Egg	(Yolk)		0.8 ¹
	(Albumin)		0.06 ¹

¹ Data from Reference 263.

² Data from Reference 190.

³ Data from Reference 13.

X. NATURAL OCCURRENCE

1. Distribution

Apparently, PABA is as widely distributed in nature as most of the factors of the vitamin B complex. Yeast, from which it was first isolated (188, 200), is believed to be by far the richest source. PABA's presence in most tissues, fluids, and elimination products of the body (13, 257, 283) is established. At least three forms are apt to occur, namely, "unbound PABA", *i.e.*, PABA in the free form; "bound PABA", *i.e.* PABA associated with carriers, such as proteins, or PABA combined with other compounds, possibly peptides or amino acids; and "conjugated PABA", *i.e.* acetylated PABA. The accompanying Tables I-VI summarize the data reported to February 1, 1944. Total PABA was usually obtained after hydrolysis and was found to be far in excess of unbound PABA particularly in animal tissues but not in all the materials analyzed. Crystalline and non-crystalline enzyme preparations were noted (284) to contain bound PABA as impurities rather than as an integral part. It may be that

its determination in crystalline proteins is a useful means of detecting foreign matter which must be extremely high in PABA if it is to carry all that is present.

TABLE IV
PABA in Vitamin B Complex Materials

Preparation		Total PABA	Unbound PABA
		<i>p.p.m.</i>	<i>p.p.m.</i>
Yeast	(Cake)	4.0 ²	3.6 ²
	(Baker's)	5.6 ²	
	(Brewer's)	9-59 ¹	6-61 ¹ , 103 ² , 50-150 ⁴
	(Autolyzed)	12 ¹	7 ¹
	(Extract)	156 ¹ , 38 ⁵	157 ¹
Rice	(Bran Concentrate)	9 ¹	3 ¹
	(Bran (Galen B))	16 ¹	2 ¹
	(Polish (Labco))		14 ³
Wheat	(Whole)	0.8 ²	0.25 ²
	(Germ)	1.8 ²	0.5 ² , 1.0 ³
	(Middlings)		0.5 ³
Molasses		0.3 ²	0.2 ² , 0.01 ³
Biotin Concentrate	(Smaco 200)	6250 ¹	460 ¹
	(Smaco 1000)		770 ¹
	(Smaco 5000)		660 ¹

¹ Data from Reference 263.

² Data from Reference 190.

³ Data from Reference 13.

⁴ M. Landy, personal communication of July 30, 1943.

⁵ Data from Reference 188.

TABLE V
*PABA in Bacteriological Reagents**

Product	Unbound PABA	Product	Unbound PABA
	<i>p.p.m.**</i>		<i>p.p.m.**</i>
Beef (Extract)	0.07 [0.15 (T)]	Gelatin	0.013
Malt (Extract)	0.74 [2.2 (T)]	Neopeptone	0.11
Yeast (Extract)	157	Peptone	0.19 [0.4***]
Yeast (Autolyzed)	7.4 [12.0 (T)]	Proteose-peptone	0.37
Egg (Dried Whole)	0.36	Proton	0.06
		Trypton	0.43

* Difco.

** Data from Reference 263.

*** Data from Reference 13.

T = Total PABA.

2. Sulfonamide-fastness

Some species of microorganisms, such as *Staphylococcus aureus* and *Pneumococcus*, but neither *Escherichia coli* nor Group C *Streptococcus*, were observed (285) to release into the medium an antagonist which is elaborated in greater quantities by sulfonamide resistant pneumococci than by the parent strain. Sulfonamide-fastness, produced *in vitro* and *in vivo* with various strains of staphylococci (286), was postulated (287) to be due to the synthesis of drug inhibitors, such as PABA, by the organisms. Indeed, the antagonist is largely, if not entirely, PABA (288), since sulfonamide resistant strains of *Staphylococcus aureus* synthesize seventy times more PABA than do parent strains of the same organism. The resistant bacteria were observed to produce a hundred times more PABA than the average amount made by a variety of organisms representative of twenty bacterial genera (155). The quantity synthesized was con-

TABLE VI
PABA in Enzymes*

Preparation		Total PABA	Unbound PABA
		<i>p.p.m.</i>	<i>p.p.m.</i>
Catalase	(Beef Liver)	19	4.3
Muscle Extract	(Rabbit)	23-25	1.7
Phosphorylase		13	2
Polypeptidase	(Yeast)	130	6.6
Rennin	(Crystalline)	19	1.0
Urease	(Jackbean)	21	1.9

* Data from Reference 284.

siderably in excess of the minimum amount required for reversal of the inhibitory action of the quantity of sulfonamide to which *S. aureus* is resistant. It seems, therefore, that the staphylococcus cell undergoes fundamental changes in its PABA metabolism when becoming drug resistant. Thus, the phenomenon of sulfonamide-fastness in *S. aureus* is explained by the development of the ability to synthesize PABA in excess of the normal metabolic requirements as a result of continued exposure to sulfanilamide type compounds. *In vitro*, *in vivo*, and particularly clinical observations showed conclusively that sulfonamide resistance, once established, is retained indefinitely, and that increased PABA synthesis is associated with sulfonamide-fastness, suggesting the former to be the cause of the latter.

Confirmatory evidence was obtained (289) with ten resistant strains of staphylococci shown to produce a substance which inhibits the anti-staphylococcic action of the sulfanilamide type drugs and which was found

to have several of the properties of PABA. An interesting reproduction phenomenon was observed, namely, the strains which gave little or no inhibition of bacterial growth produced a water-soluble yellow to deep brown-orange pigment in the presence of a sulfonamide. It is indicated that this pigment is derived from PABA. Apparently, sulfonamide resistant strains of staphylococci reproduce readily in the presence of sulfanilamide type drugs because of the synthesis of significant amounts of PABA. After maximum growth is attained, the latter is changed from a colorless state to a yellow-brown pigment. A yellow pigment was also observed (290) in cultures of Tubercle bacilli of Strain No. 607 of the American Type Culture Collection when grown in the presence of 1:1,000 to 1:3,000 concentrations of PABA. At first, the color noted was bright yellow, then the cultures became brownish. The pigment was found to be insoluble in ether, chloroform or petrol ether, but very soluble in concentrated acetic acid and phenol. It is not identical with riboflavin, formed in considerable amounts by the bacilli under the same conditions; however, it may be a metabolite derived from PABA. This substance does not seem to be involved in the major reaction resulting in the yellow, orange, or red color production observed (290a) in *Escherichia coli* cultures grown in the presence of sulfonamides. However, it may enable the organisms to produce nitrite and acid in the presence of quantities of sulfanilamide type compounds adequate for the production of visible color.

Resistance in certain species, *e.g.*, *E. coli*, may not be accompanied by enhanced PABA production. A gram negative soil bacillus was described (291) as being capable of oxidizing 10 mg. of PABA in 100 ml. of medium in 24 hours. It was subsequently found (292) to interfere with the activity of the substance in human serum which inhibits sulfonamides and to produce an antagonist which, though not identified as yet, is not PABA (293).

C. Conclusion: Physiological Importance

From a pharmacological point of view, PABA may be considered to be a detoxified aniline. Since it does not act like pigments do and since no results were obtained in cases of faulty pigmentation from its local or topical application, its physiological effect is not that of an aniline-like dye. However, the question whether it is to be considered one of the B-vitamins, can be answered.

By definition, a vitamin B complex factor is

- a) a natural constituent of yeast, liver, and/or cereals.
- b) water-soluble.
- c) a growth-promoting substance for bacteria, yeasts, fungi, and/or molds.

- d) a co-enzyme or activator of enzymatic processes.
- e) physiologically effective in minute amounts.
- f) a substance which causes a deficiency disease when lacking in the diet.

The data presented in the preceding chapters seem to justify the conclusion that PABA is one of the vitamin B complex factors: it occurs in nature as universally as and side by side with most of the B-vitamins; it is about thirty times more soluble in water than riboflavin; it is a growth factor for a variety of microorganisms; its rôle as a phenolase activator has been suggested; it has been claimed to be clinically effective in amounts comparable to those employed in choline and inositol therapy; deficiency symptoms resulting from its absence in the diet have been described. However, it may be argued that PABA *per se* is not a vitamin, since the amounts necessary to show effectiveness experimentally and clinically are by far greater than the ones ingested in a normal diet. Nevertheless, it has been suggested (VII, 2) that PABA as found in natural products may possibly be more efficacious than the simple aromatic amine by itself. The PABA-peptide, *p*-aminobenzoyl-*l*-glutamic acid, although not known to occur in nature, has been reported (294) to have 8 to 10 times more anti-sulfonamide activity than equimolecular amounts of PABA *per se*. To date, the high potency of the peptide could not be confirmed experimentally, but further work will undoubtedly yield PABA derivatives with more clinical effectiveness than has the simple aromatic amine.

As recently as April 1943, Hogan and Kamm (295) remarked that the status of PABA as a vitamin still is indefinite. Nevertheless, the American Medical Association (102) commented on PABA as a vitamin in relation to human gray hair in July of the same year. In a 1944 review of the present status of the vitamin B complex, Elvehjem (296) stated that the vitamin-like property of PABA has been demonstrated and that the simple aromatic amine can undoubtedly produce certain effects in the human, but he is inclined to believe that its action is indirect. Although the U. S. Food and Drug Administration (297) prescribed in 1941 that a product designated as a "vitamin B complex preparation" should furnish demonstrable amounts of PABA, the same administrative agency (298) made known about two years later that there was no convincing evidence for PABA to be a food essential for animals or humans. Indeed, PABA occupies as peculiar a position as do some of the B-vitamins, such as choline (299). It may not be classified as a vitamin for animals capable of synthesizing it. It may be considered to be a vitamin for all species for which it must be supplied preformed in the diet in order to satisfy tissue demands. According to Williams' (300) thesis on vitamins, the significance of PABA in the intestinal physiology of the animal is just as great whether it is an indispensable food constituent or is manufactured within body tissues.

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A Critique of the Etiology of Dental Caries

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I. INTRODUCTION

In this study attention has mainly been confined to the factors that govern the initiation of caries. After the carious process has been started, its course is fairly well known to be one of dissolution of enamel and dentin by the activities of acid-forming bacteria thriving mainly on carbohydrates. Immunity to caries of certain teeth in some individuals that are attacked in others is the main stumbling block to the acceptance by some of the chemico-parasitic theory.

The critical point in the etiology of dental caries is the change from sound to carious enamel. That change is here considered as an event microscopic in space and infinitesimal in time. If it does not occur predisposing factors are of no importance; if the agents which form a cavity after the initial breach is made are absent, or are too feeble, caries may be said never to have existed.

The selection of topics for discussion has been made for the reasons here detailed. The author has been baffled by the cryptic data of many papers and hence has sought the cause and has proposed a remedy, namely, the keeping and disclosure of the data in a more finely divided state. Caries in rats has been reviewed, with respect to its etiology, as an economical guide to study of caries in man. The chemico-parasitic theory of caries has been examined with special reference to the initiation of caries as distinct from promotion. Plaque has been given attention because this feature of oral environment is most related to initiation of caries, and present studies of plaques represent a return to the study of the whole flora of the mouth. A brief review of the literature of the antiseptic power of saliva is presented because initiation of caries occurs in the medium of that fluid or at least in proximity to it. The subject of fluorine and caries is admitted because of its real significance in practical prevention of dental caries and because it puts to the test structural and local environment theories concerning caries.

II. ON RECORDING THE DATA OF DENTAL CARIES

1. General Considerations

The close, critical examination of the literature of dental caries is difficult because so much of the reports is of subjective nature. Undoubtedly the investigators have seen conditions which have justified their conclusions but, except by a case history procedure, they have no means of reporting them. Consequently the reader is largely left without particulate data.

Most biological data can be reduced to a single figure, such as an arithmetic mean and its standard error as an index of dispersion, without serious loss. However, since the data of dental caries *relate to conditions in different kinds of teeth*, expression in a single figure constitutes large and irretrievable losses of information.

2. A Working Hypothesis of Etiology

The management of data indicates that the ruling concept of the etiology of dental caries is a *single chain of events* for all cavities. This *a priori* conclusion results in no distinction being made in kinds of cavities. Therefore, the caries status of an individual, or of a community, is frequently reported

as a single number, that is by some type of caries index. If, in contrast to the above idea, an *a priori* concept of the *unique etiology of each cavity* were substituted, a different management of data would at once be necessary.

This concept proposes that no two cavities are exactly alike because of slight differences in the intensity of positive and negative causal factors that have occurred in the long history of each tooth. It suggests that each cavity has the individuality and unique characteristics of a human finger print.

This proposed substitution of a concept of unique, for one of common, etiology is equivalent to suggesting that the approach to the dental caries problem be made with an open rather than a closed mind. It asks that conclusions be drawn on an *a posteriori* basis with respect to the data.

It is patently impossible to determine qualitatively and quantitatively every factor that has played its part in determining the exact status of a cavity at any given moment. Certain factors must be grouped and short periods of time integrated to a longer one on an *a priori* basis. Specifically, for example, in a large population group, segregation of data can be made on racial, sex, and age bases but individual variation within these groups must be assumed to follow some type of distribution. Just what this pattern of variation is may also be assumed but its approximate correctness is to be asserted only by *a posteriori* considerations.

The above is a practical modification of the concept of the unique etiology of dental caries. It simply proposes to do what is possible by ordinary means to gather the data on factors which may influence the course of caries. But the important requirement of the concept is that *the data should be kept as finely divided as possible*. This demand for subdivision of data relates to both the *data of causes* and the *data of effects*.

The impression should not be left that the assumption of common etiology of caries has been rigidly followed by those who attempt to reduce the incidence of dental caries to a single figure. In many cases in which a rate of some type is given, the data of incidence of caries in the molars have been separated from those of caries in the anterior teeth, or even the caries experience of a single tooth type, such as mortality of first molars, may be discussed. This practice represents a trend to the concept of the unique etiology of caries.

3. Caries Indices

For the purpose of developing a practical mid-ground in the assembly and analysis of the data of dental caries to the end chiefly that etiology be clarified, a brief examination of some caries indices is presented.

There are generally three bases upon which to report caries incidence. These are (a) the number of subjects affected; (b) the number of teeth show-

ing caries; and (c) the number of dental surfaces attacked. Also, quite generally, these data are reported as rates and too frequently the primary data, from which the rates are derived, are omitted in whole or in part (for example, see p. 298).

In the unique case of no caries these three systems are in agreement but at no other point can one be transformed accurately to another.

In the first system a subject with a single small cavity is ranked equal with one having extensive caries. This defect is too obvious for any serious use of such a system for the study of the etiology of caries.

The comparative efficiencies of the whole-tooth and the surfaces-of-teeth systems can be roughly judged by considering how they would be used in studies of the relation of heredity to caries, in comparison of rate of progress of decay, and in the determination of the relative incidence of interproximal and occlusal caries of molars.

Morelli (159) set up a system in which the index was

$$\frac{\text{Number of teeth in mouth}}{\text{Number of carious teeth}}$$

and a subscript was used to show the number of carious teeth. Thus $J = 1.53_{19}$ meant, that of 29 teeth, 19 were carious.¹

Entin (77) criticized the index of the Türkheim school,

$$\frac{\text{Number of carious teeth}}{\text{Number of teeth present}}$$

as not accounting for unerupted and missing teeth. His index was

$$\frac{\text{Number of intact teeth}}{\text{Number of teeth erupted'}}$$

a number which ranges from 0.0 to 1.0. Both Morelli and Entin (78) noted that their indices were discontinuous functions and tabulated the series of values.

Bodecker and Bodecker (38) pointed out that in Entin's index a subject with a number of teeth with small cavities would be reported as worse off than one with fewer teeth badly decayed. They proposed a system based on 100 susceptible areas so that the index would be directly the number of such areas observed to be decayed. In effect they selected 100 areas by

¹ Morelli gave 32₀ as the index number for a caries-free mouth. He did not discuss 32₁, which is the index number for a single carious tooth in 32 teeth.

Morelli's index numbers are in a tangential series. Those who make a practice of adding and averaging numbers without consideration of whether or not they are in arithmetical series should contemplate the results of determining a mean Morelli index by adding and averaging some values obtained by his method.

excluding teeth infrequently decayed (lower incisors and the four canines) and the third molars, because of late eruption. The upper incisors and premolars were considered as having 4 susceptible areas, the lower premolars as having 5, and the 1st and 2nd molars as 6 each. Unerupted teeth were included as non-carious and lost teeth as three points, subject to change because of case history. Crowns were recorded as three points and teeth "which serve as abutments for bridges may be rated lower." Re-decay around margins of fillings, erosion, and periodontoclasia were considered for evaluation.

In the case of the deciduous teeth all 20 were considered, the 5 susceptible surfaces giving the desired base, 100. The caries index of a mixed dentition offered difficulties.

A "Caries susceptibility index" was the annual increase in the "life caries index." "Relative age index" is comparison of the individual with "the average index of a group of persons of the same age, sex, and of the same vocation." "Relative age caries-susceptibility index" is the relative annual increase in caries index compared with the group. These latter two indices require the pertinent statistical accumulations.

Bodecker (36) deemed it advisable to modify the index of Bodecker and Bodecker by inclusion of all 32 teeth. As this totalled 160 surfaces, he provided a table for reduction to a base of 100, that is, each cavity counted 0.625%. Information was given on how to score fillings involving two surfaces, two cavities on one surface, and other details.

Bodecker (37) expanded the base of his caries index to 180 surfaces, retained the idea of a quotient but did not consummate the operation. Thus 28 carious surfaces were expressed as an index of 28. The increase from 160 to 180 was because of recognition of both mesial and distal pits in molars and premolars as separately subject to decay.

Day and Sedwick (63) used $\frac{\text{Number of cavities}}{\text{Number of teeth present}}$ as an index and presented separate values for upper and lower teeth as well as for the whole mouth.

Klein and Palmer (113) introduced the term DMF meaning "decayed, missing, or filled." Further definition was "with respect to a child, that the mouth contains 1 or more actively decayed, 1 or more filled, or 1 or more missing permanent teeth." Klein, Palmer, and Knutson (114) extended DMF to describe surfaces of teeth. Rate of DMF per 100 was calculated.

The Bodecker index and Klein and Palmer's DMF have been widely used. Sandler (179) has provided an equation for deriving the Bodecker index from DMF. Such a conversion would be justified only for the purpose of comparison with some existing body of data and not as a routine measure.

Rosebury, Karshan, and Foley (176) scored fissure caries in decalcified

sections of one of the lower jaws of rats with a scale of 4 sizes of cavities. Later (177) they expanded their score to 10 stages of caries. Originally they designated their scores by plus marks but in their revision changed to numerical values as "the numerical system also permits averaging to obtain a comparative index for each animal and each group." They obtained a value "by dividing the sum of the values for that tooth by the number of affected teeth in the group rather than of all the teeth. The index value was obtained by dividing the total score for the group by the number of animals in the group."

Cox, Dodds, Dixon, and Matuschak (56) introduced a system of scoring dental caries in rats based on sites of decay. This method differs from the basis of the Bodecker system in that the designation of the sites to be examined was on a purely empirical basis; the Bodecker index originally was mainly based on 5 theoretical surfaces per tooth and later was modified along empirical lines.

Cox and coworkers provided a brief description of (a) cavities scored in unsectioned teeth, designated "occlusal" and (b) for the fissure caries of Rosebury, Karshan, and Foley (176). Reference to these descriptions is by number. They proposed recognition of three sizes of cavities, namely, 1, 2, and 3 and *warned against adding and averaging such numbers as they are not in a true arithmetic series*. A better designation would have been A, B, and C. It was proposed to use *number* of cavities as an index of susceptibility to initiation of caries and *size* as the measure of rate of progress. The arithmetic procedure for analysis of these data were presented and discussed. The scoring of caries was recorded on punch cards (see Figs. 1 and 2) to facilitate management of the data, particularly for the operations of correlation.

The scoring system of Cox, Dodds, Dixon, and Matuschak (56) has met with some favor as it has been used by the workers in at least three different laboratories (McClure and Arnold, 124, Armstrong, 6, Dale and Powell, 60) but in each case with modification. This latter is an indication of its flexibility and also that there probably is dissatisfaction with some of the descriptive phase.

However Cox, Matuschak, Dixon, Dodds, and Walker (57) in a study of the effects of fluorides on caries incidence lost the advantage of their system of scoring by deriving a single figure as an index, that is number of cavities per animal. If they had given the frequency of decay for each pair of their 17 empirical sites it is possible that they would have found that certain of the sites of decay would have been protected by preeruptive fluorine to a significantly higher degree than others. They may have found that some areas derived no protection. Such findings, if obtained, would have illuminated the relation of fluorine to caries in a much more incisive way than did the single figure index type of statement.

4. A Proposal for the Recording of Dental Caries

The fundamental flaw of a caries index is that it adds together unlike things. For example, an occlusal cavity in a molar is a different entity from an interproximal cavity in a lateral incisor. Also, two subjects, described as having 3 decayed teeth each or 5 surfaces affected, could not be regarded as having comparable conditions unless the same 3 teeth or the same 5 surfaces are decayed. Expression of such findings as indices is only further obscuration.













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DENTAL CARIES IN RATS													
NUTRITION FELLOWSHIP OF THE BUHL FOUNDATION, MELLON INSTITUTE													
SERIES		LITTER		RAT		SEX		DIET					
BORN		KILLED		DIED		FINAL WEIGHT		FINAL LENGTH					
FATHER		MOTHER		TOTAL COUNT		CARIES INCIDENCE		ATYPICAL					
SOURCE		GROWTH RATE		OBSERVER									
REMARKS													
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<div style="display: flex; flex-wrap: wrap;"> <div style="width: 50%;"> <p>1 2 3 -9</p>  <p>3</p> </div> <div style="width: 50%;"> <p>10 -3 2 1</p>  <p>6</p> </div> <div style="width: 50%;"> <p>1 2 3 -31</p>  <p>9</p> </div> <div style="width: 50%;"> <p>32 -3 2 1</p>  <p>12</p> </div> </div>													
LEFT				RIGHT				LEFT				RIGHT	
UPPER												LOWER	
TOTAL COUNT													

FIG. 1
The Scoring of "Occlusal" Caries.

The main purposes of dental caries records are (a) to serve the needs for individual patients, (b) to provide material for the study of etiology, (c) to make group comparisons such as on sex, race, age, and regional bases, (d) to determine response to preventive or therapeutic measures, (e) to make estimates of cost of treatment of large groups.

For the individual nothing can replace the exact record of all minutiae of his dentition. No surfaces can be neglected because of infrequent decay in groups of individuals. How his index compares with that of his

"community," an age, sex, and occupation group, is somewhat academic in comparison with his concern for the probable life of each of his natural teeth.

The other four uses of dental records, indicated above, require large numbers of subjects. It is necessary that the methods of recording caries data be as uniform as possible to facilitate use. To this end the following proposal is made.

LITTER												RAT NUMBER																		
Occlusal Incidence____S. D.												Fissure Incidence____S. D.																		
Upper						Lower						Upper						Lower												
Left						Right						Left						Right												
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
1																														
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20																														

Incidence_____

 Mean Cavity Size_____

 Block No._____

 Remarks:

Scorings_____

 Observers_____

Total Score_____

 Mean Score_____

Symbols: o—1st grinding of tooth
x—tooth out
F—jaw finished
⊙—interproximal

FIG. 2
The scoring of "fissure" caries.

Establish for human caries a strictly empirical basis of description of caries to be recorded *for group studies*. Keep the number of empirical sites to be observed to a practical minimum, excluding, say, those which on past observations are known to be found in less than 5% or 2% of human mouths at, say 20 years of age. Thus the lingual surfaces of all lower teeth would not be examined in group studies. The lower incisors would be omitted. But the mesial and distal pits of the molars would be given independent statuses. "Untreated" caries, fillings, crowns, unerupted and missing teeth would be recorded as such and kept separate in the data and analyzed separately unless combination with other forms is justified.

Different stages of cavitation may be recorded, particularly for untreated cavities, but not over three classifications should be used and these should be non-numerical so as to discourage misuse. The classifications should be assigned to each individual site of cavitation, though, in description, it is likely that grouping can be made. For example, stage A for the distal pit of the lower 1st molar may be, within the limits of any practical description, similar to stage A for the mesial pit of the upper first molar. But stage A of caries in the mesial surface of the upper central incisor cannot be described at all accurately by stage A of pit and fissure decay.

The data of such a system are to be recorded solely on the basis of counting the number of each class, whether decayed, missing, or other. They are not to be combined with other data until by a posteriori study such addition is found justified.

The primary data may be re-expressed as rates, preferably as probability,² but for use in comparisons the data must be available in its original form.

When the data obtained by counting any or several classes of defects by carious attack are to be used for study of a possible difference between two groups, application of the χ^2 test is indicated. Fisher (83) has shown the derivation of a convenient formula for the calculation of χ^2 in the 2×2 table, which arises in the simplest test of the independence of classification, and Yule (212) has prepared a table for estimation of the probability of the independence. The solution of more complex problems is indicated by Fisher (84).

III. EXPERIMENTAL DENTAL CARIES IN RATS

A major difficulty in the study of the etiology of dental caries has been the lack of an experimental animal susceptible to the disease. Caries has been observed in many animals but a routine means of producing caries at will has been lacking in all except the rat. The recent demonstration by Arnold (13) of caries in the hamster gives an additional species that may provide welcome confirmation of findings on man and rat.

1. Studies of Etiological Significance

The observation by McCollum, Simmonds, Kinney, and Grieves (126) of caries in the albino rat and the discovery by Hoppert, Webber, and

² Empirical probability has an advantage in that it never "expands" the data of counting. Its basis is unity—the basis of counting. When 100 is used, as in per cent, or 1000 as in per mille, if less than the number in the base is counted the data are expanded to the appearance of greater precision. On the other hand, if considerably more than the base number are counted, there is a "loss" of precision.

The figures of probability, per cent, and per mille are identical but vary in their relation to the decimal point. Probability starts with the decimal point and may be extended as far as the data justify; per cent and per mille must be extended to reach the decimal point no matter how they may be lacking in precision.

Canniff (104, 105) of a method of initiating caries in that animal opened a field for experimental exploration of the etiology of caries.

Hoppert, Webber, and Canniff (104, 105) undertook to produce caries in rats by certain dietary procedures. Their methods were not effective. They examined the teeth of rats on a stock ration and found a very appreciable incidence of caries. This particular stock ration consisted of

Yellow maize meal	60
Whole-milk powder	30
Linseed meal	6
Alfalfa meal	3
Sodium chloride	1

They found that if the maize were ground to pass a 60 mesh sieve that no caries resulted in the rats, the ration with coarse corn meal produced a high incidence of caries.

These findings of Hoppert, Webber, and Canniff (104, 105) led to a critical examination of dental caries in rats as previously observed by others. It was revealed that in all previous reports of caries in rats, in which the diets were clearly described, that coarse cereal particles had been fed. Bunting (43) said in reference to the observations of Hoppert, Webber, and Canniff, "These findings, which have been corroborated by others, have negated practically all previous animal experimentation in which the fineness of the food was not taken into consideration, and has clearly shown the fallacy of many conclusions drawn from such experimentation." This criticism by Bunting applies to the findings on rat caries, say, prior to 1933. These include work which seemed to show that a deficiency of phosphorus in the diet of the rat resulted in caries, Shibata's (182) studies on various sugars as initiating caries, and Marshall's (138, 139) conclusions respecting vitamin A deficiency as resulting in caries in the rat.

Shelling and Asher (181) studied the relation of cereal particles to rat caries and concluded: "In the rat there is no correlation between the Ca and P intakes, the concentrations of Ca and inorganic P of the serum, and caries-immunity, and caries-susceptibility." Also "Results on the experimental production by this means of caries-like lesions in rats are not necessarily applicable to the etiology of dental caries in human beings."

In a paper immediately following that of Shelling and Asher, Rosebury, Karshan, and Foley (176) described three distinct types of dental lesions in rats caused by coarse cereals. They described (a) fractures of the cusp and (b) dissolution of the tooth originating at the summit of the cusp where the dentin is normally exposed in the rat tooth. They excluded these two types of lesions as not being "comparable to human dental caries." But (c) the third type, called by them "fissure caries," originated at the base

of the sulci by penetration of the enamel, spread along the dentino-enamel junction, extension into the dentin, and finally production of active pulpitis. As a secondary effect, the undermined enamel collapsed at some stage of the development. They set up a system of scoring these cavities as to size and described the stages to which they assigned "values" of +, ++, +++, and +++++.

Rosebury, Karshan, and Foley said: "In rats the action of large particles in producing fissure caries may be explained—if we assume that dental caries arises by bacterial action on food particles retained at sites about the teeth (*italics by the reviewer*)—by postulating a process of forcible impaction-wedging—of such food particles into favorable regions. Such forcible impaction may act (a) by perforating the enamel cuticle and bringing the food mass into intimate contact with enamel, and thereafter (b) by resisting the normal mechanisms that remove or replace food masses less effectively impacted. Thus, if true, is a new principle; and while its application to human caries is of course uncertain, it may well be deserving of consideration in this regard."

In the matter of "fracture" lesions, Rosebury, Karshan, and Foley said: "The most common site of these lesions, in the mandible, was the large subdistal cusp of the first molar, which seems architecturally to bear the brunt of masticating stress. Such cavities, when fully developed, may be seen to occupy a position directly under what had been the center of the subdistal cusp; otherwise, as we have noted, they cannot be distinguished from cavities resulting from fissure caries. All such gross lesions in this work have been classed as fracture cavities. Obviously although the progress of this lesion simulates true caries in its secondary stage, its manner of inception differs greatly both from fissure caries in rats, and from dental caries in man."

Bibby and Sedwick (25) concluded: "Evidence in practically all lesions indicated that the cavities originated as fractures of a portion of a cusp or fissure wall. About 96% of cavities were found in mandibular first and second molars. The explanation is offered that the peculiar morphology of these teeth permits them to be easily fractured by forces from the occlusal surface. Development of such fractures is promoted by presence in the diet of coarse hard particles. It is suggested that heretofore fracture-lesions have been confused with caries, although the conditions resemble each other only in late stages."

This criticism by Bibby and Sedwick was based on (a) the comparatively infrequent occurrence of caries in the maxillary molars of the rat and (b) the morphology of the sulci of the first and second mandibular molars, namely, an overhanging posterior wall which they concluded was subject to fracture.

It is quite possible that under certain circumstances, depending on the strain of rat used and dietary matters relating to the formation of the teeth, that coarse cereal caries would be entirely restricted to the first and second lower molars. Bibby and Sedwick probably observed rats from such a set of factors. But close inspection of some of the reports of caries in rats appearing in the literature prior to 1933 and subsequent statements reveal that caries is quite common in the upper first and second molars of rats on coarse cereal diets. In fact Finn and Hodge (82) in 1941 said, "The maxillary first molar is most frequently involved and . . . there is evidence that the teeth of the upper jaws are more often carious." It may be pointed out that Finn and Hodge found only seven teeth in 124 rats with fissure caries and regarded four of these lesions as artefacts. But they said, "Perhaps the long duration of the experiment permitted other fissure caries to coalesce with or simulate occlusal cavities and thus prevent identification."

The paper of Rosebury, Karshan, and Foley (176) appeared in the number of the *Journal of Dental Research* just preceding the one which carried the study by Bibby and Sedwick. The latter were thus not influenced to examine thoroughly the effects of cavity development at the base of the sulci. It has been the author's conclusion from inspection of thousands of "sections" of rat teeth prepared by the method of Cox and Dixon (55), that in the great majority of cases fracture of the posterior wall of the sulci of the lower first and second molars is a result of undermining of the enamel by caries originating at the base of the sulci. This conclusion is not altered by rat caries pictured in the literature as in practically all such cases cavitation is too far advanced for any conclusions to be drawn as to the site and initial nature of the "lesion." The author has also observed frequent cases of caries in the floor of the sulci mesial to the subdistal cusp of the first molar. The cavities have been seen in all stages of development prior to loss of the cusp by gross fracture. It is the opinion of the author that loss of this cusp is, contrary to the idea advanced by Rosebury, Karshan, and Foley (176) (see page 265), a result in practically all cases of fissure caries.

King (109) noted the forward inclination of the posterior walls of the sulci of the lower molars of rats and indicated that fracture of such walls initiated coarse cereal caries. He also advanced theoretical reasons for the origin of fissure caries by wedging of coarse cereal particles and forcing the walls apart resulting in fracture. Rosebury (169) did not concur in this explanation and pointed out that radiopaque areas appear in the enamel in very early fissure caries and that these cannot be explained on a traumatic basis.

In addition to the three types of lesions discussed above, fissure, fracture,

and cusp summit dentinal caries, at least three other types have been observed. Grieves (96) and Klein and McCollum (112) described interproximal caries, and Rosebury (171) and Weisberger, Nelson, and Boyle (203) found proximogingival caries. It is of especial interest to note that the gingival caries of Rosebury were produced by hard pilot biscuit and not by coarse cereal; the gingival caries seen by Weisberger, *et al.* were in rats deprived of salivary glands.

Cox, Dodds, Dixon, and Matuschak (56) described an "opaque" type of lesion occurring on the distal-buccal angle of the first lower molar and on the approximating mesial-buccal angle of the second molar. Similar lesions occurred at the buccal end of the floor of the mesial sulcus of the lower first molar and in a similar position in the main sulcus of the lower third molar. Cox, *et al.* also found occlusal lesions occurring at the point of fusion of segments of the main cusps of the first and second molars both upper and lower.³

Sognaes (189) showed further the importance of the coarse cereal particle in rat caries. He extracted the upper molars of young rats. No caries developed in 100 days in such rats on coarse cereal. If extraction of the teeth was effected after the rats had been on the ration for 30 days, caries resulted but in lesser degree than in normal rats. Extraction of upper teeth on one side reduced the incidence of decay on the opposite lower side, presumably because of lowered masticatory efficiency.

2. Some Results of Studies of Caries in Rats and Their Significance

Although it is necessary to disregard certain *positive* findings with respect to the etiological significance of certain nutritive factors if coarse cereal particles were present in the caries-producing diets (43) there are *negative* findings in the absence of coarse particles that retain their significance. Specifically it has been found that a high content of fermentable carbohydrates in the diet of the rat for long periods of time, with or without the addition of bacteria, does not induce caries (Rosebury and Karshan, 173; Agnew, Agnew, and Tisdall, 2; Lilly, 116; Lilly and Grace, 117; Johnston, Kaake, and Agnew, 107; Rosebury and Foley, 172; Day, Daggs, and Sedwick, 62; and King, 109).

But Rosebury and Karshan (174) found that sucrose "tended to increase the incidence of caries on both the rice and corn diets, but inconsistently and to a degree that is not statistically significant." The additions of sucrose were not high, being 8, 18, and 28%. Rosebury (170) further commented: "Although sugar alone does not produce caries, addition of

³ The "opaque" type of caries occurs in areas 11, 12; 17, 18; 21, 22; and 33, 34 shown in the chart in Fig. 1. The lesions at incomplete cusp fusion are in areas 1, 2; 3, 4; 7, 8; 15, 16; and 27, 28.

sugar to a coarse corn or rice diet usually increases its caries-producing effects." It has been the experience of the author and his coworkers that glucose or sucrose, fed to rats on a cereal-free ration *after* caries has been induced by coarse cereal particles, cause the cavities to increase in size in comparison with a control ration containing uncooked corn starch. The sucrose level in these experiments was 66%, with equivalent amounts of glucose and starch in the other rations.

The above observations, with respect to fermentable carbohydrates, suggest that there is a difference in the conditions necessary to *initiate* caries and those needed to *develop* cavities. Glucose and sucrose, and presumably any carbohydrate that can give rise to acids in the mouth, will promote enlargement of cavities but fail to cause their initial appearance.

Elsewhere it is shown that fluorides fed precruptively to rats increase the resistance of their molars to coarse cereal caries (p. 289), and that reduction of flow of saliva in rats increases caries attack (p. 281). As these findings are in accord with observations on man it appears that coarse cereal caries in rats is a means of study that can provide direction for the study of dental caries in man, if proper allowance is made for species difference in respect to the initiating factor. The mechanism of initiation of caries in the rat is less obscure than the process in man.

IV. MICROORGANISMS AND DENTAL CARIES

1. *Origin of the Chemico-Parasitic Theory with Especial Reference to Structure of Enamel and the Initiation of Caries*

The chemico-parasitic theory of W. D. Miller undoubtedly explains the excavation of cavities in enamel and dentin, but Miller, himself, had doubt concerning the initiation of caries. He evidently considered structure of enamel as influencing both initiation and rate of caries progress. However, structure is now generally disregarded in caries etiology but as indicated below there is ample evidence for retaining the concept of structural resistance to the initial attack of caries in any fair minded study. Also there is given evidence that studies that keep the facts of caries particulate are most likely to reveal the most of etiology. Suppression of bacteria by selective media is a form of suppression of facts and it is encouraging to note that investigators are returning to the viewpoint that the entire flora of the mouth must be studied in the bacteriology of dental caries.

Robertson (167), in 1835, considered dental caries "a process of putrefaction or fermentation in the several parts of the teeth best adapted for their reception." Buehlmann (42), in 1840, reported seeing thread-like forms in saliva, on teeth and especially in tartar. Erdl (80), in 1843, observed microorganisms in films removed from teeth. Ficinus (81),

in 1847, considered caries a putrefactive process related to organisms he called "Denticola."

Leber and Rottenstein (115) were the first to find microorganisms in carious dentin. They described a leptothrix which they believed penetrated enamel, after it was softened by acids, and later expanded and destroyed the tubules of dentin mechanically. They did not consider the leptothrix as forming the acid to soften the enamel.

Underwood and Milles (199) showed sections of decayed dentin containing "micrococci, oval and rod-shaped bacteria." In the surface layers the bacteria were very numerous but in deeper decay only an occasional organism was found. They concluded: "We consider that caries is absolutely dependent upon the presence and proliferation of organisms. That those organisms attack first the organic material, and feeding upon it, create an acid which removes the lime salt, and that all the differences between caries and simple decalcification by acids is due to the presence and operation of germs. This view we propose to call the 'septic theory'." On the basis of this theory they proposed and practiced antiseptic procedures in the treatment of caries.

Miller (145, 146), in two papers covering much the same material, excluded neutral substances, such as sugar and ammonium chloride, as solvents of tooth substance and considered only acids as effective. "There is no difficulty in accounting for the source of the acids concerned in the caries of the teeth," he wrote, and illustrated the ready formation of organic acids by bacteria acting on bread. He showed that when sound dentin was placed in a mixture of bread and saliva it was decalcified. Meat, on the other hand, yielded alkaline products and no decalcification of dentin.

He demonstrated the presence of leptothrix and cocci forms of bacteria in the tubules of decayed dentin and showed that they were less numerous in the deeper portions. Also he found a zone of dentin partially decalcified by the acid products of the microorganisms but as yet not infected.

Miller found that dentin exposed to oral microorganisms without carbohydrate food for four months was not decayed. The microorganisms gradually died and no acid was formed. However, with bread added, acid formation could be detected in 6 or 8 hours and the presence of lime from tooth substance within 20 minutes.

He concluded: "The first stage of caries of the teeth, *i.e.*, the extraction of the lime-salts, is for the most part caused by those acids which are generated in the mouth by fermentation." "In the separate tubules is frequently to be seen a gradual change from leptothrix threads to long bacilli, from long to short bacilli, and from the latter to micrococci." He concluded these forms "stop up the canaliculi, and necessarily lead

sooner or later to the death of the dentinal fibrils." He considered 3 stages of caries (a) decalcification, (b) infection, and (c) putrefaction of devitalized dentin. However he did not wish to be understood as saying "acids or pathogenic bacteria, or putrefactive bacteria, or all together, are the sole and only cause of decay of the human teeth." But "there is not a single case of caries in which microorganisms do not play some part, and that in most cases they play a very important part."

Miller (147) stated his credo:

"1st. Caries of the teeth is not entirely of a parasitic nature.

"2nd. The first stage of dental caries consists of a softening of the tissues of the tooth.

"3rd. In effecting the softening, acids, particularly those generated by fermentation, often play a very important part.

"4th. Whether microorganisms are or are not concerned in the production of the first stage of caries, or whether any other agent is concerned in the process (be it in the form of a simple solvent, or be it a pathological action on the part of the tooth itself) are questions which certainly, as yet, cannot be satisfactorily answered.

"5th. Destruction of the enamel may take place quite independently of microorganisms.

"6th. The second stage of caries, devitalization, and breaking down of the softened tissue, is entirely parasitic, and is inaugurated chiefly, if not wholly, by the elements of the *Leptothrix buccalis*, while later on various other fungi may take part in the decomposing process.

"From this it may be seen that I am a believer in acids, a believer in micro-organisms, and a believer in an unknown cause; that is, I believe that there are agents active in the production of caries which are yet to be discovered."

Miller (148) stressed the difficulty of obtaining sections of enamel to show caries but succeeded in preparing 150 sections from 125 different teeth. He observed "in the beginning of the carious process" a slight depression on the surface of the enamel with and without masses of *Leptothrix buccalis*. Beyond this "the enamel will almost always be very perceptibly discolored, as though acted upon by some agent producing effects undistinguishable from those of acids." In practically no case did he observe microorganisms in the enamel unless comparatively deep penetration had occurred.

Similarly in enamel decayed by spread of caries along the dentino-enamel junction, he could find only an occasional organism. He said: "I am driven to the conclusion that there is as yet no sufficient ground for assumption that micro-organisms play any more than an unimportant part in the caries of enamel." However, this is not to be interpreted that

Miller did not believe that acid produced by bacteria did not decalcify the enamel; the organisms simply were not *in situ*. In dentin he found fungi in the outer half of altered tissue.

Miller (149) showed that if a hole is bored into dentin, and saliva and bread allowed to ferment therein that subsequently putrefaction will take place in the hole after the bread and saliva are removed. No putrefaction occurs if the decalcification is not first effected. Teeth bound together in such a way as to leave a space at the necks about 2 mm. wide developed decay when placed in bread and saliva, the site of decay being at the point of lodgment of fermenting material. Miller showed by chemical analysis that carious dentin, from various sources, was decalcified.

Miller (150) summed 18 propositions as a result of his studies in two years on an estimated 8000 teeth. Briefly they are:

1. Amylaceous or saccharine food and saliva generate acid.
2. There is constant formation of acid in the mouth because of retention of food in cracks, pits, and fissures of teeth and between teeth.
3. The degree of acidity depends on length of time of exposure.
4. A cavity of decay containing amylaceous or saccharine food always has an acid reaction.
5. "The extent to which any tooth suffers from the action of the acid depends upon its density and structure, but more particularly upon the perfection of the enamel and the protection of the neck of the tooth by healthy gums. What we might call the perfect tooth would resist indefinitely the same acid to which a tooth of opposite character would succumb in a few weeks."
6. An occasional neutral or alkaline reaction in a cavity does not mean that acid did not produce the cavity.
7. A systemic condition which withdraws lime salts from a tooth or lowers its density or weakens the union of organic and inorganic matter renders it more liable to decay.
8. "Strong acid and corroding substances brought momentarily into the human mouth, may give rise to lesions of the enamel at points where the ordinary agents alone could never have begun."
9. *In vitro*, caries, simulating in macro details natural caries, can be produced with acid mixtures such as found in the mouth.
10. Carious dentin is decalcified but roughly inversely porportional to depth.
11. Destruction of organic material follows, not precedes, decalcification.
12. Mouth fungi do not act directly in decalcification. Further study is needed of their acid producing activities.
13. Fungi produce great changes in decalcified dentin and finally reduce it to a mass of debris and fungi.

14. "The invasion of the microorganisms is always preceded by the extraction of the lime salts."
 15. Destruction of organic material is accomplished by fungi.
 16. "Inflammation can hardly be looked upon as a very important factor in caries of the teeth."
 17. "Caries of the enamel is purely chemical, the decalcification resulting at once in the complete dissolution of the tissue."
 18. Caries of cementum runs a course similar to that in the dentin.
- Of the above 1, 2, 3, 4, 6, 8, 9, 10, 11, 13, 14, 15, 17, and 18 may be regarded as conclusions reached by Miller as a result of direct observation on extracted teeth as well as those *in situ*. However, 5, 7, and 16 may be regarded as opinions. Number 12 is offered tentatively.

Miller's experiments in regard to structure of teeth and caries were as follows: He "broke up several teeth, perfectly free from caries, but of different densities, into fragments of various sizes, and put the pieces in a mixture of saliva and bread." He kept the mixture at 37°C. for three months, with renewal of the bread and saliva five times. The attack, producing typical caries, was variable. "In two cases, evidently owing to a defect in the structure, the teeth were attacked on the cusps and reduced to a powder; where the acid had penetrated through the enamel, its effect was seen to spread out in all directions in the dentine; where the enamel was very hard and dense, without crack or blemish of any kind, it had not even lost its lustre."

"This experiment shows clearly enough what a vast difference the structure (density) of the tooth makes in its resistance to decalcification, and offers an explanation of the question why all teeth do not decay alike. A dense tooth, covered with enamel, intact at every point, would probably resist for years the action of an acid saliva, to which a soft, defective tooth, would succumb in a few weeks.

"It also shows that the process of decalcification may go on in cracks and fissures too fine for food to penetrate, in fact anywhere, where saliva holding starch or sugar in solution may find access, and be retained even by capillary attraction, there to undergo fermentation."

The facts of varied attack of caries on enamel under artificial conditions are clearly stated by Miller but he did not demonstrate any differential structures associated with either freedom from or susceptibility to decay. His inclination to believe in enamel structure as a factor in caries is clearly evident.

It may be said that Miller was influenced by the then current belief that soft teeth are more susceptible to decay than hard teeth. The same may be said of his Proposition No. 7 as he offers no experimental work to support systemic decalcification of teeth. As to No. 16 he simply found no evidence of inflammation, that is his findings were purely negative.

Miller (151) in the first section of a paper continued through 5 numbers of Independent Practitioner, described *Bacterium acidi lactici* isolated from carious teeth. In the 2nd section he proved that a mixture of fresh saliva and starch, when properly incubated, forms lactic acid, which he isolated as zinc lactate. In the 3rd part he described the various involution forms of the bacterium ranging from cocci to thread types. He distinguished α - and β - forms. His 4th section dealt with the effects of dental filling materials and antiseptics on dental fungi. In the last part he isolated three more fungi in studies of pure cultures. All five were acid formers without gas formation and "They may consequently all be looked upon as factors in the decay of the teeth."

Miller summed his work to date as:

1. He had shown microorganisms present in dentin and stated there was no claim for priority as Leber and Rottenstein (115) had earlier found the same.
2. There is a zone of softened dentin that contains no microorganisms.
3. Chemical analysis shows decalcification in carious dentin.
4. Maintained from first, but without proof, that acids produce decalcification of dentin.
5. Proved the presence in the mouth and in carious dentin of acid-producing fungi.
6. Produced artificial caries.
7. Showed the effects of antiseptics on oral acid-producing fungi.
8. Described the characteristics of the acid-producing fungi.

Miller (152) described culture methods for microorganisms and stated that he had isolated 22 different fungi from the human mouth. In 1889 (153) he published his findings in book form with an edition in English in 1890 (154). These books are generally cited for the chemico-parasitic theory of Miller. His observations on resistance of teeth to microorganisms, presumably because of structure, are generally ignored.

Marshall (139) wrote: "You will recall that Miller said in 1883 and again in 1892, that he was not prepared to state positively that dental caries was caused by microorganisms; but what he did say was that microorganisms played a large part in the etiology. This statement holds true today as then." The foregoing is not a quotation from Miller. What he did say after reviewing a number of facts apparently contrary to his chemico-parasitic theory of caries, including absence of bacteria in enamel, was "these facts, I say, and others of like nature, suggest the thought that the first stage of caries may have other causes than those of a parasitic nature, and make us hesitate before we cast up everything in favor of bacteria."

But Miller's disciples were not as cautious as he, either then or now. For example a quotation picked at random (Allan, 5) is "The actual presence of

an acid as a commencement or initiative step in the process of decay being acknowledged Miller shows us whence it comes, names it, and points out the little organism at work manufacturing it." This statement, which is typical in its connotation of many that have been made since, asks that *initiation of caries by acids be acknowledged without proof*. The enlargement of cavities by acids from bacteria can readily be acknowledged as there are abundant data to support that idea; there is only faith, engendered by a constant repetition of such phrases as that of Allan "acid . . . initiative step . . . being acknowledged," that substitutes for facts.

2. Specific Organisms

On the basis of the Miller theory, bacteriologists have sought a specific organism as the cause of caries. Miller (157) said of the number and kinds of bacteria in the mouth that it "is one of the most difficult problems with which the dental bacteriologist has to grapple. . . ." He considered whether "processes of fermentation go on differently in different mouths." He stood on his previous conclusions (156) that though the saliva of immunes may develop a little less acid than that of susceptible persons when in contact with carbohydrates, the difference is too slight and variable to account for the marked difference in susceptibility to caries.

McIntosh, James, and Lazarus-Barlow (127) considered it "necessary to find microorganisms which were capable of softening enamel, which process histo-pathological research has shown to be the initial lesion in caries." They considered that the microorganisms that could form acid sufficiently potent to soften enamel must also be able to live in such a medium. Consequently they first sought the pH necessary to produce a significant whitening of enamel. They concluded that pH of 4 or less met their requirement. Their most successful broth was at pH 3.5. With it they isolated from carious dentin two strains of bacteria which they called *B. acidophilus odontolyticus* I and II. These strains produced a mean final pH of 2.75 when inoculated in glucose broth of pH 7.6. They produced artificial caries in teeth, resembling natural caries, by immersing teeth in broth cultures of these bacteria. Their illustrations show attack on enamel at specific sites.

Clarke (52) isolated a streptococcus from teeth in early stages of decay and called it *S. mutans*. It was not so aciduric but highly acidogenic. He found it decalcified enamel more readily than the *B. acidophilus odontolyticus* of McIntosh, James, and Lazarus-Barlow (127) and considered it associated with beginning caries. "*B. acidophilus* could be isolated only from teeth in which cavities had formed, or in which the foci of caries were shallow. . . ." Of 40 cases of fissure caries *S. mutans* was isolated from dentin in 27 and was in pure culture in 8 cases. Bunting and Palmerlee (45) con-

firmed McIntosh, James, and Lazarus-Barlow by use of acid media. They found "*B. acidophilus* is present in every initial lesion of dental caries." Maclean (120) confirmed Clarke (52) and criticized Bunting and Parmerlee because their acid media did not give *S. mutans* a chance to grow. Enright, Friesell, and Trescher (76) concluded: "The only microorganism commonly found in the food debris, in direct contact with progressive caries of the enamel, that can tolerate and produce additional acid in an environment below pH 5.0, is a lactobacillus..." They did not discuss the work of Clarke (52) and Maclean (120).

Fosdick and Hansen (87) discussed acid production from carbohydrates as a function of an enzyme system following Meyerhof's scheme rather than the work of a microorganism, *per se*. They considered that certain enzymes may be derived from one organism and others from different organisms. For example, they found that an oral yeast yielded a mean solution of enamel equivalent to 22 mg. of calcium. Under the same circumstances *B. acidophilus* failed to dissolve enamel. However the yeast and the *B. acidophilus* acting symbiotically averaged 45 mg. solution of calcium.

Anderson and Rettger (7) concluded that microorganisms other than lactobacilli and streptococci, particularly yeasts and leptothrix forms, may be of importance in dental caries.

Bibby and Hine (23) found by direct counting "extreme differences occur in the flora of carious cavities." They found a high percentage of cocci, especially Gram-negative types, suggesting "these organisms are causative agents" in caries. The comparatively low frequency of finding lactobacilli raised doubts concerning their importance in caries.

Harrison (98) found streptococci the predominant organism on the surfaces of non-carious rat molars, about half the strains being acidogenic. In rats with caries the surface flora consisted mainly of lactobacilli (99). The organisms isolated from deep carious tissues were mainly acidogenic streptococci (100). Lactobacilli were found in enamel caries but not in deep dentinal lesions. "The apparent relationship of lactobacilli with initial caries and of streptococci with advanced lesions of the dentin" was discussed.

Fosdick (86) found lactic, pyruvic, phosphoglyceric, and possibly butyric and hexosephosphoric acid produced in the natural fermentation of glucose in saliva in the presence of calcium phosphate. Fosdick and Wessinger (89) showed that oral yeasts are capable of degrading glucose to phosphoglyceric, pyruvic, and lactic acids. They remarked that the primary ionization constants of these acids are 3.8×10^{-2} , 5.9×10^{-3} , and 1.38×10^{-4} , respectively, and "Since the former acids are formed first and are stronger than lactic acid, it would appear that they are the compounds that actually decalcify the teeth." Fosdick and Starke (88) found the rates of different

enzymatic actions different for *B. acidophilus* compared with oral yeast and suggested, to explain the symbiotic effect of these organisms, phosphorylation of glucose by yeast, joint production and hydrolysis of phosphoglyceric acid and the reduction of pyruvic acid to lactic acid by acidophilus.

Bibby, Volker, and Van Kesteren (27) estimated that lactobacilli make up less than 0.1% of the strongly acidogenic organisms of the mouth and, since streptococci are 1000 times more numerous and are more rapid acid formers, "that lactobacilli give rise to no more than .025 per cent of the acid formed by the action of salivary organisms on carbohydrates." They found that the acid production was affected in variable degrees by the presence of tooth substance in the culture media. They observed "that as long as tooth substance was present in the bacterial cultures the pH did not fall below pH 5." *This observation should materially lessen the importance of aciduric considerations in judging whether or not a given organism can be considered in caries etiology.*

Florestano (85), though stating "no correlation could be made between the absence or the presence of dental caries and the amount of acid formed by microorganisms," found "aciduric streptococci and staphylococci were isolated consistently from both carious and non-carious individuals." He considered those microorganisms were secondary to their food supply, that is carbohydrates, in dental caries. He found that "oral streptococci and staphylococci may produce as much acid as lactobacilli." Of lactobacilli as specific agents in caries he said: "This now classic theory is hardly reconcilable with the data presented above and with observations by other workers."

3. Plaques

Acid in saliva and acid formation by bacteria free in saliva are inadequate to explain attack on enamel. Accordingly "others conceived of a 'film' or 'plaque' on the tooth surface which confined acid to the tooth surface and prevented neutralization by the saliva" (193). It is interesting to note that Miller (155) did not accept this addendum to his chemico-parasitic theory.

Black (28) in 1886 announced the isolation of oral cocci which caused a peptonized broth with 2% sugar to gel rapidly. Williams (205) found microorganisms in dense, felt-like films on enamel with both normal and what he called beginning carious surfaces. He concluded in characteristically uncompromising fashion "that if the environing conditions of the teeth are such as to favor the development and activity of acid-producing bacteria, and if those bacteria are permitted to become attached to the surface of the enamel, it is doomed, although it may be the most perfect ever formed. On the other hand, if the environing conditions are not present the worst enamel will not decay."

Black (31), commenting on Williams' work, quoted from Black (29) in regard to caries fungus: "It has power of itself to penetrate enamel or dentin, and the acid must precede it continually, as we find by observation it does. Growing against the side of a tooth it will often form this gelatin and protect itself from removal until it works its way through, and it crowds itself into the tubules and fills every available space as it goes." He also said: "The one thing necessary to the beginning of caries is the formation of such a gelatinous microbial plaque in a secluded position where its acids may act without too frequent disturbance. . . ."

Miller (155) expressed doubts concerning plaques as essential for the initiation of caries. He pointed out that plaques are frequently present where caries seldom appears such as on lingual surfaces. Also he found carious areas in enamel with no covering film although he said that did not mean that no plaque had existed. By eosin staining, he found films on teeth to be very prevalent. The films particularly were found where natural cleansing did not occur, and these sites are frequently attacked by caries. But he questioned the interdependence saying: "If there is any interdependence at all, it is just as natural to suppose that the softening of the surface of the tooth produced by a beginning calcification furnished a more ready opportunity for attachment of the film."

Lothrop and Gies (119) wrote of mucin plaques: "Any influence of mucin as an *adhesive* medium, and of bacteria and fungi as corrosive and *enamel-puncturing* agents, in the initiation of the carious processes, are doubtless exercised to their greatest degrees during periods of sleep."

Bibby (21) found a brown pigmented plaque to be similar in physical and chemical nature to salivary calculus and that "the incidence of caries in 100 cases having brown plaques was little over one-half the average in 1000 patients of corresponding ages, attending the same clinic, but having no brown plaques."

Dobbs (74) immersed freshly extracted teeth in dilute organic acids and observed decalcification. The positive test was sufficient softening to show a scratch from a hard point. He found variable decalcification by 5 fermentation acids, variability of attacks on teeth from the same person, differences of resistance of teeth from different subjects, and lack of uniformity on the surface of a single tooth. He found resistant areas were covered with plaques.

Dobbs removed plaques from teeth with 5% HCl containing some $KAl(SO_4)_2$, a procedure that probably produced some alteration of properties. He tested diffusion of acids and bases through such plaques finding, KOH and lactic acid readily passed through but that Na_2HPO_4 did not diffuse. He concluded that "acids formed beneath plaques are but slightly affected by saliva."

"Dental caries was not found beneath all plaques." Under thick plaques he found whitened areas and reasoned that limitation of carbohydrate supply and "accumulation of decalcified enamel" had inhibited further action.

Dobbs produced artificial plaques on teeth by immersing cleaned teeth in a bread and saliva mixture for 8 hours and allowing them to dry in air for 16 hours. The process was repeated for 12 days. He considered such a process may occur *in vivo* with the dry mouth stage occurring in sleep.

In his discussion Dobbs wrote: "Whether plaque is essential for the progress of decay has not been established, but it is apparently essential for the initial lesion of caries." The truth of this assertion depends on the definition of its terms. "Carbohydrates are necessary for fermentation and acid production, therefore necessary for the initiation of dental caries." Fermentable carbohydrates do not *initiate* caries in the rat though caries is *promoted* in that species by carbohydrates (see p. 268). "The passive resistance of enamel can not be the controlling factor in immunity to caries." However enamel formed with an optimum of fluorine is resistant to decay (see p. 289). The truths boldly written by Dobbs in 1932 must be modified or discarded in consideration of knowledge accumulated since then.

Dobbs also wrote: "It appears that the passive resistance of enamel is a factor in controlling the rate of decalcification and, to a lesser degree, the susceptibility of teeth." This belief that passive resistance of enamel to caries begins only after initiation of caries is of uncertain origin in facts. It is part of the lore of dental caries such as "A tooth for every child" and "A clean tooth never decays."

In 1936, Blayney, Kesel, and Wach (35) reported the beginning of a series of studies of association of bacteria with plaques. Direct smear preparations of plaques were made and examined for bacterial types. Those associated with caries always had *L. acidophilus*; non-carious types "contained any form except typically short rods and coccobacilli." They found a high per cent of agreement for acidophilus between smears and saliva cultures. Noyes (163) collaborated with Blayney, Kesel, and Wach (35) by comparing clinical conditions with diagnosis by smears. Associated with carious surfaces he found 73% of the smears gave positive diagnosis; with non-carious surfaces positive smears were found for 13.3% of the cases.

Stephan (192) measured the pH of 211 plaques removed from surfaces "clinically intact and readily accessible." A micro-colorimetric method for pH estimation was used. He found an average⁴ pH of 5.9 with a range from pH 4.6 to 7.0. The pH of plaques from areas recognized as carious

⁴ This is an arithmetic mean of pH values and not the pH corresponding to the arithmetic mean of hydrogen ion concentration.

averaged 5.2, and 10 of 38 were below pH 5.0 which Stephan considered as the critical point below which significant attack on enamel begins.

Stephan (193) adapted the antimony electrode to the determination of the pH of plaques *in situ*. He found that the pH of labial and buccal plaques fell an average of about 2.0 units after a 1 minute rinse of the mouth with 10% sucrose or glucose solution. In 45 minutes to 2 hours the original pH of the plaque prevailed. Boiled starch solution caused a drop of 1.5 units. A 1% lactose solution reduced pH only 0.3 units compared with 1.1 reduction caused by 1% glucose.

Bradel and Blayney (40) instituted a study of the organisms associated with plaques from carious and non-carious areas. The difficulty of recognizing when caries begins was noted as they said: "It usually requires many months and sometimes years for the lesion to reach the stage where it can be detected macroscopically." In 409 cases classified as "immune," "relatively immune," "relatively susceptible," and "susceptible" they found 7.5, 13.9, 35.3, and 81.6%, respectively, with plaques containing flora which they associated with caries. The clinical condition was determined independently.

Blayney, Bradel, Harrison, and Hemmens (34) said: "The clinical aspect of the caries problem constitutes a definite stumbling block in the determination of the etiologic factors of this disease and presents a challenge to the keenest powers of observation of the investigator and clinician." In their study of plaques from proximal surfaces of premolars they accumulated series of bite-wing roentgenograms which enabled them to trace the development of cavities. They found this system of roentgenograms "will reveal lesions months before they can be detected by the methods usually employed." This statement directly suggests that carious lesions exist long before they can be detected by any macroscopic means and that consequently the term "caries-free" is always suspect.

Blayney, *et al.* found that in plaques the incidence of lactobacilli increased from 7% at 10½ months prior to discovery of caries on a surface to 43% at the time of definite clinical evidence of decay, and to 77% 9 months later. These data suggest the association of lactobacilli with early enamel caries but by no means constitute a proof that they initiate the condition. There is no way to determine just when initiation occurs.

The technique of Blayney, *et al.* with respect to (a) time, (b) study of specific areas on the tooth surface, and (c) the organisms should yield distinguished results on the relation of microorganisms to tooth decay.

V. THE CARIES-INHIBITING ACTION OF SALIVA

Saliva is related to certain features of this review—bacteria, both free and in plaques, formation of plaques, acid attack on enamel, and fluorine.

Accordingly, a brief survey of the effects of saliva on caries is presented with especial reference to its antiseptic effects. Re-mineralization of enamel, though obviously related to structure, is not discussed.

Robertson (167) said: "The saliva having a natural tendency to occupy the lower part of the mouth, the teeth there placed are constantly bathed with this fluid, which, by its property of dissolving the particles of food, is in a great degree calculated to remove them; or by its decidedly antiseptic qualities, prevents the process of putrefaction."

Hugenschmidt (106) plated various organisms in gelatin with Chamberlain filtered saliva and the same after heating to 60° C. to destroy bactericidal substances similar to those found in blood. He found torula growth more restricted by heated saliva, no action of saliva of either kind on sarcina, results similar to torula for staphylococci, and no effect on cholera bacteria. He concluded: "The bactericidal action of the saliva appears to be *very problematical*." He discussed the volume effect of saliva being of the opinion that a relatively dry mouth was conducive to oral infection. He indicated a belief in an acid buccal mucus secretion with bactericidal properties but made no tests. He found no bactericidal action from potassium sulfocyanide. The chemiotactile property of saliva for phagocytes was found to be associated with bacteria.

Miller (156) found no antiseptic properties in saliva passed through a Chamberlain filter or any effect on acid formation. Potassium sulfocyanide at 1:1000, or eight times that found in saliva had so slight an effect on bacterial growth and activity that Miller dismissed it as ineffective. Mucus separated from saliva fermented and putrefied more intensely than clear saliva. He found variable results of fermentation rates of salivas of persons immune and susceptible to caries, somewhat favorable to the immune subjects, but he was not prepared to draw definite conclusions. Miller confirmed Hugenschmidt (106) in finding no bactericidal heat labile substance in saliva.

Miller (157) made these following observations on saliva and dental caries:

- (a) A strong flow of saliva is associated with low caries attack and, *vice versa*, diminution of flow is observed in extensive caries. "The amount of saliva which flows from the glands during a dental operation must not be taken as a necessary index of the amount normally secreted."
- (b) He could find no definite relation of viscosity of saliva to caries.
- (c) Experimental demonstration was made that more bacteria developed in a medium initially alkaline than in one with acid reaction. The final result may frequently be more acid from the alkaline start. So he concluded reaction of the saliva itself had little if anything to do with caries.

- (d) The carbohydrate of saliva is negligible as a source of acid compared with carbohydrates of foods.
- (e) The antacid properties of saliva, due to calcium salts, is nil.
- (f) The antiseptic power of saliva is negligible.
- (g) Miller reaffirmed his conclusion (155) "that bacterial plaques are essential neither to the beginning nor to the progress of caries, nor does their presence necessarily result in the production of caries." He found teeth were decalcified, if anything, more rapidly in areas from which he removed plaques.

Cheyne (49) found that rats from which the salivary glands had been removed were much more susceptible to dental caries and, in fact, developed caries on rations which were without effect on normal rats. Weisberger, Nelson, and Boyle (203) described similar results.

Trimble, Etherington, and Losch (197) found in 107 subjects a mean stimulated secretion of saliva of 34 cc. per 15 minute period, normally distributed in a range from 9 to 68 cc. The results were reproducible when repeated after an interval of two weeks and after 6 months. In a 12 month period 57 subjects with less than average rate of secretion developed 28 new smooth surface cavities; the remaining 50 with greater than average saliva secretion rate developed 15 new cavities.

Bibby, Hine, and Clough (24), by means of "wells" in agar plates containing saliva, demonstrated that "growth of 110 of 169 strains of bacteria tested was prevented by human saliva" but those least affected were organisms from the mouth.

The possibility that fluorides in saliva may inhibit caries is discussed on p. 290.

VI. ENAMEL STRUCTURE AND DENTAL CARIES

1. *Studies Concerned with Enamel Structure*

Black (30) in 1895 said: "I have chosen to confine the examinations for density, proportion of water, lime salts, and organic matter, to the dentin, as best expressing the character of the tooth as a whole." Black's data on specific gravity, per cent of water, per cent of lime salts and per cent of organic matter, elasticity and crushing strength of dentin with relation to age, sex, carious condition, and vitality of the pulp, fill 24 full pages—one of the finest bodies of data in the literature of dental caries. However, in drawing his conclusions, Black did not use the word "dentin." He used "teeth." He said: "Differences in the strength of the teeth have no influence as to their liability to caries. Differences in the density, or in the percentage of lime salts in the teeth, have no influence as to their liability to caries." "Caries of the teeth is not dependent upon any condition of the tissues of the teeth, but on conditions of their environment."

It is largely upon these conclusions of Black that structure and chemical composition of the *enamel* are dismissed as factors having to do with determining whether or not caries will start. Black's conclusions are justified for caries in the *dentin* with respect to the factors that he studied. It may be pointed out that Black chose dentin as the portion of the tooth to be studied because he was interested in the relations of dental tissues to filling materials.

Truman (198) offered two main criticisms of Black's work, namely, on his method of choice of the teeth and on the selection of dentin as best representing the tooth. He said: "While this may possibly have been the most convenient and best course to pursue, it leaves the coat of mail, the enamel, entirely out of the question, and this, the protective shield, is certainly an important factor in the inquiry."

Black (31) as well as Williams (206) replied to the criticism by Truman but neither undertook any defense of the use of dentin to represent the whole tooth, and, in particular, enamel. Black (32) in 1899 still ignored the criticism of Truman as he wrote: "caries is not dependent upon the quality of the teeth as to their structure or their perfect or imperfect calcification."

It is an interesting sequel that it was Black (33) who first observed that caries was less frequent in mottled enamel than in "normal" teeth. The discovery by Churchill (51) and by Smith, Lantz, and Smith (187) that fluorine from water causes mottled enamel; the analyses of enamel by Armstrong and Brekhuis (10) that show an association of freedom from caries with a certain level of fluorine in the enamel; the observation by Dean, Jay, Arnold, McClure, and Elvove (69) that teeth need not be mottled to enjoy freedom from caries, have certainly reopened the question of chemical composition of enamel and the relation to caries.

Miller (157) selected hard and soft teeth by the criteria of yellow and bluish, respectively, and deciduous teeth compared with those from elderly subjects. Sections were prepared and exposed to a saliva and bread mixture. He found a reduced rate of decalcification of the *dentin* from the so-called hard teeth but the difference was insufficient to account for immunity to decay. Secondary dentin in one section was attacked less effectively than primary dentin. Miller said: "It is a very common thing to find one of the bicuspid decayed to the pulp while the approximating surface of the other shows only the first beginning of caries. This phenomenon is difficult to account for unless we suppose that the teeth have different resisting powers."

Miller (158) in a continuation of his 1904 paper, examined the resistance of enamel to acid decalcification. He pointed out that enamel is far more resistant to decalcification than dentin and that "chemical destruction of

enamel takes place to a very great extent from within outward and is very materially assisted by mechanical agents." He said: "The external intact smooth enamel surface offers a most stubborn resistance to the action of weak acids and far greater resistance than the internal surface," and illustrated this fact experimentally. He also illustrated that "abrasions, bruises, cracks, or any other defects, natural or artificial, very sensibly diminish this resistance." Some of the resistance is due to the enamel cuticle, and he warned against its destruction. He showed that the cuticle remained tenaciously in place after caries was well established.

Another property was one "which appears still more important than the behavior of the enamel cuticle. The external surface of the enamel seems to be protected by a thin layer or crust which offers a marked resistance to the action of acids in comparison with the deeper layers. Wherever this crust, which is only a fraction of a millimeter thick, is mechanically removed, the acid at once acts far more rapidly upon the enamel than otherwise." The effect was illustrated by sections, particularly by one in which a cut was made parallel with the enamel prisms.

Miller also showed by exposure of sections to acid that secondary dentin, transparent dentin, and self-healed dentin were more resistant to decalcification than normal primary dentin.

With respect to an outer caries-resistant layer of enamel, Hodge and McKay (101) found: "It is interesting and even amazing to note that the extreme outer shell of enamel has a maximum hardness on the same scale represented by 2,050." The middle portion of enamel was rated at 910 and the inner layer at 330. Thewlis (196) has shown that enamel increases in radiopacity from the dentino-enamel junction to the outer surface and that there is apparently a peak at the surface independent of the Mackie effect.

Bodecker and Ewen (39) found 179 cases of "unilateral caries" in 516 full mouth X-rays, unilateral caries being defined as decay affecting only one of two approximating tooth surfaces. These cases were those in which variation in environment were eliminated, and resistance must necessarily have resided in the teeth. They discussed this rather frequent phenomenon as indicating a structural resistance to caries.

2. Some Nutritional Studies

Mellanby (141) reported the beginning of what proved to be a long study of the relation of structure to caries saying at that time "if the enamel on all parts of the crowns of teeth is abundant and sound, and if the teeth are adequately spaced, then such teeth are less likely to be attacked by caries and other diseases." This statement has always been objectionable to the local environment theorists either as a premise or a conclusion. Mellanby selected the dog as an experimental animal and showed that restriction of

calcifying agents, particularly vitamins A and D resulted in formation of teeth grossly hypoplastic in both enamel and dentin but could not show that dental caries resulted in the dog because of these poor structures. Her experiments on dogs and on children are somewhat cumulatively presented in 1934 (142), a publication that Weaver (202) thoroughly analyzed. His main objections to the conduct of the work, analysis of the data and the conclusions are: (A) Association between two conditions has been taken as a causal relation, incidentally, a very frequent error. (B) There are several conflicts between theory and facts especially in reference to permanent teeth. (C) The findings can be interpreted in other ways, particularly in respect to treacle which in some experiments was used as a control on olive oil and cod liver oil feeding. (D) The theory is not in harmony with facts on (a) the liability to caries of different surfaces of teeth; (b) the incidence of rickets; (c) incidence of caries in New Zealand, Australia, and South Africa; (d) asymmetry of caries on opposite sides of the mouth; and (e) the relative liability to caries of vital and pulpless teeth.

In the matter of rickets in relation to dental caries, Friel and Shaw (91) have pointed out that in Johannesburg actinic rays received from the sun are about 10 times as much as in England, and rickets is non-existent. But of 600 Johannesburg children 560 had caries. On the other extreme, Taylor and Day (194) found in the Kangra District of the Punjab, Northern India, "a very low incidence of dental caries and hypoplasia." A diet and health survey had "shown a high incidence of rickets and osteomalacia and definite vitamin D, calcium, and phosphorus deficiencies in the diet of most of the population there." Presumably the deficiencies prevailed over long periods and could be reasonably assumed as concurrent during tooth formation. They gave 10 case histories with severe rickets. Among these there were two decayed deciduous teeth, four carious permanent teeth, and two cases with four defective fissures each. Four had perfect teeth with respect to caries.

Rosebury and Karshan (175) found in posteruptive studies that corn oil, olive oil, Wesson oil, crisco, and lard were effective in reducing the caries index in rats, but paraffin oil was without effect. Vitamin D in a minimal amount exerted an independent effect in lowering the index. However they concluded "the mitigating effect of vitamin D on caries is relative and limited" and "no amount of vitamin D can be expected to prevent caries."

If there is in reality a structure associated with resistance to the initiation of dental caries it remains to be demonstrated by means of a specific structure. The evidence—histological, physical, or chemical—must be shown to be present particularly in the surface of the enamel of teeth that will not decay, and absent in those that do. The test of resistance to decay should be either the bread and saliva type test of Miller, or proof that the tooth has

existed caries-free in a human mouth for a long period of time under conditions that promote caries, such as long time indulgence in carbohydrate foods. Posteruptive effects of nutrients acting through metabolic channels are probably mainly, if not entirely, exerted through dentin and have not been considered here in etiology as concerned with initiation.

VII. FLUORINE AND DENTAL CARIES

1. Summary of the Discovery of Fluorine in Various Tissues and in Water

Fluorine is qualitatively the most important element associated with dental enamel. An excess intake of fluorine in the period of formation of enamel causes mottled enamel; an optimum intake results in a lowered incidence of dental caries; a posteruptive application of relatively concentrated solutions of fluorides reduces the incidence of new cavities. The first of these effects is structural, the second probably so, and the last possibly a bacteriostatic effect concentrated at the surface of the enamel.

In 1802, Count Morozzo was called to inspect the fossil bones of a large animal found in some shallow excavations near Rome. He concluded the bones were those of an extinct species of elephant (161). In particular he obtained a molar which he submitted to a chemist, Morichini, for analysis of its parts. Further details of the fossils were reported by Morozzo (162) with a statement by Morichini occurring therein. Morichini treated portions of the enamel and dentin with sulfuric acid and, finding that the gas emitted would etch glass, reported that the molar contained "fluoric" acid.

Morichini (160) reported his work in full and also the detection of fluorine in the bones and teeth of contemporary man. He suggested that fluorine may have a relation to dental disease but his implication was of diagnostic intent. Klaproth (110, 111) obtained samples of the elephant molar from Morozzo and confirmed the presence of fluoric acid. He suggested that it was derived from phosphoric acid.

Gay-Lussac (92) related the details of Morichini's work in a letter to Berthollet in 1805, and it was this notice that attracted the attention of the chemists of that time. Chenevix (47) combatted Klaproth's idea of the origin of fluoric acid by slow transformation of phosphoric acid. Fourcroy and Vauquelin (90) failed to find fluoric acid in enamel, in recent ivory, and in some fossil ivories, but Chevreuil (48) succeeded. The details of their procedures suggest that Fourcroy and Vauquelin had obtained the comparatively soluble calcium fluosilicate, but Chevreuil's (48) procedure produced calcium fluoride. Brande (41) of England failed to find fluorine in the enamel of human teeth possibly because the glass surfaces he exposed to the evolved gases were too extensive for him to detect the etching. Proust (166) found fluorine in the bones of a fossil elephant and in fragments of

bones and teeth of a fossil horse. Berzelius (18, 19) confirmed Morichini by finding fluorine acid in teeth and bones. He found fluorine in urine, though in very small amount, by precipitation with lime water. Thus by 1806, the chemists of Italy, Germany, France, England, and Sweden had taken a lively interest in fluorine in the tissues of animals. The interest is shown by Delametherie's (72) note on Proust's report and Gehlen's (93) editorial activity in reporting to German chemists the work of Morichini (160), Proust (166), Fourcroy and Vauquelin (90), and Chevreuil (48), and also the full publication of the results of Berzelius (17). Nicholson's *Journal in England* commented editorially on fluorine in bones in 1806 and in 1807.

Berzelius (20) found fluorine in Carlsbad waters in 1823 and estimated a concentration of 3.2 p.p.m. as calcium fluoride. Will (204) reported in a footnote the presence of fluorine in plant ashes. Wilson (208) in 1846 confirmed the presence of fluorine in urine. He found it in well waters of Edinburgh and with considerable difficulty demonstrated its presence in ox blood and in cheese. He said: "Physiologists will doubtless now be tempted to speculate on the possibility of fluorine performing some essential function in living animals." Wilson (209) reported fluorine present in ocean water, and (210) in 1850 verified his discovery of fluorine in ox blood and proved its presence in milk directly instead of indirectly by analysis of cheese. Crichton-Browne (59) in 1892 suggested the need for fluorine by women "to fortify the teeth of the next generation."

In 1842, Girardin and Preisser (94) proposed to determine the age of fossils by the amount of fluorine present. This method was examined by Daubeny (61), Middleton (143), and Smith (185) at that time and later by Carnot (46) and Wilson (211). Hoppe-Seyler (Hoppe, 103) suggested fluorine was present as a fluoapatite in bones and teeth thus giving an explanation of the accumulation of fluorine in bones, a puzzling fact to the investigators of fossils. Incidentally Morichini's discovery of fluorine in the fossil elephant molar was probably made possible only by such an accumulation. The fossil was found in a volcanic formation, and the ground waters were probably rich in fluorine.

2. Mottled Enamel and Caries

In 1901, Eager (75) reported his observation of black teeth (*denti neri*) among the natives of Pozzuoli, 5 miles from Naples. The teeth were called "*denti di Chiaie*," and Eager said because "this defect was first described by Prof. Stefano Chiaie, a celebrated Neapolitan, and bears his name."⁵

⁵ This derivation of "*denti di Chiaie*" was denied by Guerini in correspondence with McKay (136) who said it was named for a quarter of Naples in which the condition was frequently observed. The works of Stefano delle Chiaie, available to the author, reveal no reference to such teeth.

In 1915, McKay (133) reported the study by himself and Black of a condition which they called "mottled enamel," endemic in and around Colorado Springs. Their publication of details (33, 134, 135, 136, 137) marks the effective beginning of the study of the causes of mottled enamel. The outstanding features of their papers with respect to dental caries are that they associated the condition with the water supplies and "as to caries, the teeth of these children compare favorably with those of other communities where endemic mottled enamel is unknown. . . . But when the teeth do decay, the frail condition of the enamel makes it extremely difficult to make good and effective fillings. For this reason many individuals will lose their teeth because of caries, though the number of carious cavities is fewer than elsewhere."

McKay persevered in the study of mottled enamel and clung to his belief, in spite of discouragement by facts and men, that some trace element in water was the cause. In 1925, he advised change of water supply at Oakley, Idaho (128) and in 1933 (132) was able to report that mottled enamel was no longer being developed there.

In 1926, McKay (129) reviewed the evidence for the occurrence of the cause of mottled enamel in water and asked for suggestions from water works men. Among the many replies was one from Hannan (97) who wrote: "Since the enamel is essentially mineral in composition, and the water definitely incriminated, the mineral content of the water seems the probable source of the trouble. Of the mineral elements at present known to be common to both water and enamel, the chief are calcium, phosphorus, and fluorine. For our intake of phosphorus, we are independent of the small proportion found in water; the same can be asserted of calcium with perhaps a shade less certainty, a dietary deficient in this element being not altogether unusual. But when we consider fluorine, all is at present shrouded in obscurity." McKay (130) replied to the suggestions but regrettably not to Hannan's fluorine idea.

In 1925, Schulz and Lamb (180) and McCollum, Simmonds, Becker, and Bunting (125) described the effects of fluorine on the development of rat incisors, and the latter authors, in particular, mentioned "a dull, opaque white color" of the enamel and spoke in one instance of "mottled areas." McCollum, *et al.* "were led to consider whether perhaps a deficiency of fluorine in the food might lead to the formation of teeth which had poor structure and consequently possess little power to resist the agencies which lead to decay."

Bunting, Crowley, Hard, and Keller (44) stated relative to mottled enamel at Minonk, Illinois, that the percentage of children affected by caries was about the same as elsewhere but that it was limited to small pit and fissure caries of the molars.

McKay (131) advanced the idea that, because there was not more decay

in mottled enamel, that structure has no relation to liability to decay. This argument is not valid in view of the recent evidence that *slightly* mottled teeth are less affected by caries than "normal" teeth from low fluoride areas, but, the invalidation is in no way proof of the contrary.

In 1931, Churchill (195, 51) announced the discovery of the presence of fluorine in the waters of Bauxite, Arkansas, where severe mottled enamel had been described by Kempf and McKay (108). Churchill also found 2 p.p.m. or more fluorine in other endemic regions and less than that in areas free of mottled enamel. Smith, Lantz, and Smith reported in 1931 (186, 187) observations on fluorine and mottled enamel that were made in the same period as those of Churchill. They found fluorine in the waters of St. David, Arizona, where severe mottled enamel was endemic. By giving the waters concentrated to 1/10 volume to rats they produced chalky enamel of the incisors. They identified the condition with that observed by McCollum, Simmonds, Becker, and Bunting (125) and thus practically completed the etiology of mottled enamel.

Others who noted a reduced incidence of dental caries in mottled enamel districts were Ainsworth (3, 4), Masaki (140), and Erasquin (79). Ainsworth, in particular, noted that the deciduous teeth, which are very seldom mottled in endemic regions, had a reduced rate of caries as he wrote "12.9 per cent of deciduous teeth carious against 43.3 per cent in all districts." In his 1928-1929 paper, Ainsworth, of course, was unaware of the cause of mottled enamel; in his 1933 report, which was largely reprinted from the earlier paper, he added comment on fluorine with regard to mottled enamel but made no suggestion on its possible effect on caries.

Arnim, Abérle, and Pitney (11) examined 204 Indian children in regions where the well waters contained fluorine up to 2.8 p.p.m. They found white spots in 24% of 1605 permanent incisors, but no carious lesions. They, however, ignored the *lowered* incidence of caries in order to attack the idea of the probable mechanism of such lowering by saying: "The observations recorded herein add another group of teeth, structurally defective, in which the incidence of caries is not increased."

3. Direct Evidence of Anti-Caries Action of Fluorine

a) *Analytical.* An important event in the fluorine-caries problem was the development by Armstrong (8) of a reliable method for the determination of fluorine. With it Armstrong and Brekhus (10) found the mean fluorine content of the enamel of sound teeth to be 111 p.p.m.; that of carious teeth, 69 p.p.m. The difference was 11.9 times the standard error and hence of undoubted significance. They considered, with caution, a causal relationship. They recognized (9) that mottled enamel, with fluorine content over 300 p.p.m., may become carious and said: "It is

possible that caries occurring in severely mottled teeth are not entirely comparable to caries of nonmottled teeth. The fact that the relatively slight amounts of fluorine in mottled enamel are accompanied by profound changes in its properties makes it seem entirely possible, in the light of the evidence presented above, that optimum quantities of this element in enamel might be associated with an increased resistance to caries without the deleterious effects on the appearance and the structure produced by an excessive fluorine content."

b) *Experimental*. Cox, Matuschak, Dixon, Dodds, and Walker (57) fed 10 and 40 p.p.m. of added fluorine as sodium fluoride to rats during pregnancy and lactation and tested the young for susceptibility to coarse cereal caries. They found a significant increase in caries resistance, compared with young from mothers on rations with no added fluorine. The molars of the young were not mottled. All young were placed on the same caries-producing ration with no added fluoride, that is, no fluoride was given to any of the young after they were weaned at 21 days of age. They wrote: "Our evidence that fluorine aids in the formation of caries-resistant teeth, linked with the findings of Armstrong and Brekhuis (10) and of Dean, Jay, Arnold, McClure, and Elvove (69), shows that a very great reduction of the incidence of human caries can be obtained by supplying in food and water an optimum amount of fluorine during tooth formation." They concluded their data were evidence that "caries resistance can be built into enamel." The findings of Cox, *et al.* (57) relative to preeruptive fluorine have been confirmed by Norvold and Armstrong (6).

Volker (200) studied rate of solution of enamels with varying contents of fluorine at pH 4.0. He concluded: "The presence of fluorine in large amounts may decrease the solubility of the dental hard tissues. Small amounts of fluorine show no demonstrable reduction in enamel solubility. It seems doubtful that the amounts of fluorine present in slightly fluorosed teeth is sufficient to alter their acid solubility."

c) *Field Studies*. Extensive surveys of the relation of water to dental caries have been made by Dean and his associates. In the initial paper of the series (64) presumptive evidence was presented that the caries rate was low in mottled enamel regions. Four questions were raised, concerning namely (a) relation of structure to caries; (b) the significance of the fluorine content, *per se*, of the enamel; (c) the inhibitory action of fluorine on the enzymatic processes; and (d) whether or not fluorine is the only element in the pertinent waters active in prevention of caries. Dean and his coworkers, however, have confined their attention mainly to the inhibition of enzymatic action and generally with negative results.

Dean, Jay, Arnold, McClure, and Elvove (69) found the caries rate of 12, 13, and 14 year old children of Galesburg and Monmouth, Illinois, with 1.7-

1.8 p.p.m. of fluorine in the water supply was of the order of 200 carious teeth per 100 children compared with 633 at Quincy, Illinois, with 0.2 p.p.m. of fluorine in the water. The lactobacilli counts in saliva were correspondingly lower at Galesburg and Monmouth compared with counts in Quincy. They reported the results of McClure's (122) studies of salivary amylase, the values for maltose being 105.9 ± 5.2 mg. for 63 specimens of saliva from Quincy, and 108.7 ± 3.1 mg. from 82 salivas from Galesburg. McClure wrote: "It may be said with reasonable assurance that fluoride ingestion, brought about by the use of a domestic water supply containing approximately 1.8 p.p.m. of fluorine, does not change the final amyolytic activity of the saliva secreted under these conditions."

Bibby and Van Kesteren (26) found that "fluorine concentrations of less than 1 p.p.m. limit acid production by bacteria, but concentrations in excess of 250 p.p.m. are needed to affect bacterial growth." There are two important deductions to be drawn from these findings of Bibby and Van Kesteren. Cox (54) calculated from preliminary data of Bibby and Van Kesteren that their culture medium contained 0.32 p.p.m. of fluorine. Cox and Matuschak (54) found in "a single sample of stimulated saliva pooled from three children each from Galesburg and Quincy . . . 0.095 and 0.12 p.p.m. of fluorine, respectively." McClure (123) analyzed pooled salivas from a number of localities including Galesburg and Quincy, and found the fluorine level to be about 0.10 p.p.m. and within limits of the variation of his analytical method, uninfluenced by fluoride containing waters of the range of variation found in Illinois. Hence the lowest concentration of fluorine tested by Bibby and Van Kesteren (26) was approximately three times that found in human saliva. Furthermore as there is no indicated difference in concentration of fluorine in saliva between areas with high and low fluoride waters, a difference in rate of production of acid cannot be expected because of the influence of this ion. Consequently the thesis of Dean (64) that fluorides influence the course of enzymatic reactions is unsupported with respect to (a) amylase and (b) the acid producing enzyme systems of bacteria, in so far as fluoride of saliva is concerned.

The other concentration of fluorides referred to by Bibby and Van Kesteren is that "in excess of 250 p.p.m." to affect bacterial growth. Obviously there are no such concentrations of fluoride involved normally in any of the fluids concerned in dental caries. Even the concentration falls below that value in sound, unmottled enamel (10). That there is a higher concentration of fluorine in the surface of enamel remains to be demonstrated as well as the fact that it, *in situ*, is effective in limiting growth of bacteria. Hence, number of lactobacilli would seem to be unaffected by fluoride differences in either fluids or enamel, and the difference of

counts found by Dean, *et al.* (69) cannot be explained on such a basis with present knowledge.

Dean (65) reviewed the relation of fluorine to caries and expressed doubt of the significance of the results of Cox, *et al.* (57) who interpreted their findings with rats as indication of resistance built into the enamel. But Dean (65) considered then only two hypotheses, namely, the effect of the fluorine content of enamel as providing (a) acid-resistant or (b) antienzymatic material or antibacterial surface effects. However, Dean, Jay, Arnold, and Elvove (67) wrote: "Whether or not this inhibitory agent operates locally, systemically, or even involves structural or compositional factors is still undetermined." They examined children of Bauxite, Arkansas, who had been on fluorine-free water for 12 years after change of the water supply from a 14 p.p.m. level of fluorine. They found the older children with mottled enamel, which had been formed prior to the water change, had markedly less caries than comparably aged children of Benton, Arkansas, four miles away and continuously on low fluorine water. Children born within 1½ years of the change of water were practically free of mottled enamel and had a low caries rate. A third younger group had an increased rate of caries, in fact an inversion of the usual pattern of increase of cavity incidence with age as they had more caries experience than the older children. The exposure to fluorine-free water had therefore no effect on caries. The counts of lactobacilli paralleled caries activity and not the fluorine content of the water.

Arnold, Dean, and Elvove (15) examined the lactobacillus counts of children of Garrettsville, Ohio, where a change of water supply resulted in an increase of the fluorine level from 0.1 to 0.7 p.p.m. They found no change of the *L. acidophilus* counts over a period of two years, though they considered that the period of observation was too short.

Dean, Jay, Arnold, and Elvove (68) examined the children of 8 Chicago suburban cities, and Dean, Arnold, and Elvove (66) children of 13 additional cities. The chief significance of this extension of their methods is that in 21 cities they have extended the association of fluorides in water supplies with a low caries rate to a fair certainty on this basis alone. Their presentation of the dental caries experience of 7,257 12-14 year old children plotted against the fluorine content of the public water supply should convince the most confirmed skeptic that fluorine is a significant beneficial element in dental caries.

The above association of low caries rate with fluorine in water seems to supply a practical answer to Dean's question as to whether fluorine alone or other elements are active. However, the questions relating to structure of enamel and its fluorine content remain unanswered by any part of the field work of Dean and his coworkers. Their finding of 19

times as much interproximal caries experience in the superior incisors of low fluoride cities compared with fluoride populations, and 4 times as much first permanent molar mortality, is difficult to explain on a basis of local oral environment as the sole cause of caries. If this crude separation of localities of caries attack were refined to the presentation discussed on page 26, *et seq.*, it is not improbable that other striking differences, unexplainable on the bacteria-as-initiating-agents theory, would appear.

Deatherage (6) has provided evidence of another type of the beneficial value of fluorine in caries. He found in army selectees in Illinois, ages 21-28, a total caries experience rate of 581 carious teeth per 100, in 77 men who had lived the first 8 years of their lives in communities with fluoride waters. In 82 men of comparable age who spent the first 8 years in low fluoride areas and then in high, the rate was 1,077 carious teeth per 100. Later Deatherage (70) has increased the number of subjects. The data below are condensed from his table.

Fluorine and Dental Caries

	No.	Age 21-24	Age, Number, and Caries Rate		No.	Age Over 28
			No.	Age 24-28		
F in water, 1st 8 years						
F free	232	865	129	1061	88	1241
0.5-0.9 p.p.m.	67	748	41	622	47	1317
1.0 p.p.m. and over	202	613	152	793	159	811

Deatherage gave data also on those who had spent all their lives in high and low fluoride areas. The data (a) support those of Dean, (b) indicate that most of the protection is derived by the factors acting in formation of the enamel, and (c) show that protection is extended beyond 28 years of age.

Dean, Jay, Arnold, McClure, and Elvove (69) included some data of 76 children who for various reasons had not been in Galesburg for all of the first six years of their lives. They excluded these data from most of their conclusions. Cox (54) concluded that these children had a significantly higher caries rate than children of Galesburg who had been exposed to fluoride water continuously and, therefore, that posteruptive exposure to fluoride-bearing water supplies has no effect on the course of dental caries. Dean and his coworkers in subsequent studies have rigidly excluded children of varied exposure to fluorine. In so doing they have omitted the dynamic groups that would give information as to the time elements of the problem, such as duration of protection. The carious attack pattern in mouths with teeth of two different histories of fluorine during formation, would be a crucial test of the theory of initiation of caries by bacteria.

An example of how the advent of fluorine in the dental caries field may upset some fine spun theories is the case of Tristan da Cunha. The ex-

cellent teeth of the inhabitants of that isolated South Atlantic island had been explained by their diet of (a) fish, (b) potatoes, (c) raw milk, and (d) low intake of sugar. However, Sognnaes (190) found that mild mottled enamel was wide spread. The water contained only 0.2 p.p.m. fluorine so Sognnaes considered the fluorine derived from a food source, probably fish. Sognnaes and Armstrong (191) confirmed the presence of fluorine in the teeth by finding 196 p.p.m. in deciduous and 270 p.p.m. in permanent enamel, values in excess of the normal (10).

4. *Posteruptively Applied Fluorides*

Miller (144) largely inhibited the development of coarse cereal caries in rats by the inclusion of 250 p.p.m. of sodium fluoride or 500 p.p.m. of calcium fluoride in the caries-producing ration. His procedure was based upon antienzymatic considerations and he employed as an additional control a ration with 200 p.p.m. of iodoacetic acid. This latter ration undoubtedly operated by inhibiting bacterial action. As the initiating factor, the coarse cereal particle, was present at all times the net result would appear to be that caries was initiated but development of cavities was prevented. The same explanation may suffice for the fluoride series but, in view of the demonstration by Perry and Armstrong (165) and by Arnold and McClure (12) that fluorine may be acquired posteruptively by enamel, some structural or chemical explanation may be invoked. Miller's findings, confirmed by Finn and Hodge (82), McClure and Arnold (124), and others, have been the basis of the application of posteruptive fluorides in human caries. Cheyne (50) applied 500 p.p.m. of $\text{KF} \cdot 2\text{H}_2\text{O}$ in aqueous solution to the cleaned and dried teeth of 27 children at about 4 months intervals for a year. For controls 19 children were used. The incidence of newly carious surfaces was 3.09 in the experimental, and 6.04 in the controls. Bibby (22) initiated an experiment with 100 children. He treated the teeth of one quadrant of the mouth with 1/1000 sodium fluoride solution for seven or eight minutes after prophylaxis of all teeth and treatment with hydrogen peroxide and alcohol. (It is not clear whether these latter agents were used on all teeth.) Treatment was effected every 4 months. After a year, 33 new cavities were observed in the treated quadrants, and 61 in the untreated. Neither Cheyne (50) nor Bibby (22) used a solution of comparable inhibiting value on any control teeth so it is not clear whether fluorides have specific inhibiting effects on caries. Use of iodoacetic acid as a control would have indicated whether fluorides have especial dental effects, when posteruptively applied, or whether any inhibiting agent diligently used would result in a similar reduction of carious attack.

Conclusions with respect to the etiology of caries drawn from post-

eruptive experimentation with high concentrations of fluorides may be defective when applied to field studies as the latter have uniformly been concerned with low concentrations. The distinction between initiation and promotion of caries should not be obscured in evaluating the data.

5. The Toxicity of Fluorides in Relation to Possible Use in the Prevention of Dental Caries

The toxic aspects of fluorine have been dealt with by DeEds (71), McClure (121), Roholm (168), Greenwood (95), and others. It is not the intent of this study to examine the effects of the higher dosages of fluorine. The significant maximum level of fluorine from the dental caries prevention viewpoint is that which will produce no toxic effect. Consequently toxicity studies must be examined for the minimum fluorine intake that can cause any detectable signs of injury.

Shortt, Pandit, and Raghavachari (184) studied a stiffness in the lumbar region of natives of certain districts near Madras in India, said to be associated with water supplies. The condition affected adults of thirty years and over, and the final condition in persons over 40 was complete rigidity of the spine and rigidity of the joints of the upper and lower limbs. The patient eventually became completely bed-ridden. There was a heavy incidence of mottled enamel. Preliminary studies showed 0 to 10 p.p.m. of fluorine. They found the natives had learned by trial and error the low fluorine waters but attributed the choice rather to selection by hardness, which they found positively correlated with high fluorine.

Shortt, McRobert, Barnard, and Nayar (183) continued the above studies with intensive examination of ten subjects. Radiological findings were increased density of the bones in the affected areas. The majority of their cases showed renal impairment. They did not discuss this aspect as a cause or result of fluorine. Pandit, Raghavachari, Rao, and Krishnamurti (164) estimated the intake of fluorides in the Madras area of osteosclerosis on the basis of 6 pints of water per day as 5.4 mg. fluoride per day in a mild area, 9.4 mg. in a moderate area, and 13.2 to 24.0 mg. in the severe areas. (The context indicates this is fluoride ion, not sodium or calcium fluoride.) They concluded the severity of the condition had "definite relation to the economic and nutritional status of the communities." They implicated a vitamin C deficit. They found the "bone affections were fairly prevalent" in a town "with a fluoride content of only 2 p.p.m. in their water supply."

Hodges, Fareed, Ruggy, and Chudnoff (102) examined 86 subjects ranging in age from 7½ to 71 years with the fluoride content of the water supply varying from 1.2 to 3 p.p.m. (Kempton, Illinois) and 31 subjects from 18 to 78 years with fluoride at 2.5 p.p.m. (Bureau, Illinois). They concluded:

"The use of drinking water containing up to 3 p.p.m. of sodium fluoride apparently does not cause radiologically demonstrable sclerosis of the skeleton even though the water is taken for a long period of time." (Weart and Klassen, 201, from whom Hodges, *et al.*, 102, derived their figures for fluoride, make it clear that these values are in terms of the fluoride ion and not as a salt such as sodium or calcium fluoride.) Linsman and McMurray (118) reported what they believed to be the first case of osteosclerosis, observed radiologically and confirmed at autopsy, resulting from fluoride in the water, to be found in the United States. The subject had suffered a kidney injury in youth and exhibited severe anemia. The exposure to fluorine in water had varied from very low to as high as 12 p.p.m. They said: "The chronic fluoremia may have aggravated existing kidney lesions. Or it is entirely possible that, because renal impairment was present, the osteosclerosis developed as a result of fluorine retention." They recommended systematic study for osteosclerosis in regions in which the fluorine content exceeds 3 p.p.m.

The above studies indicate that significant bone injury is rarely if ever produced by levels of fluorine in water that result in improved resistance to dental caries. In each of the above studies mottled enamel was cited as evidence of the presence of fluorine in the water supplies and was apparently the more delicate sign of a toxic intake of fluorine. As the proposals of the use of fluorides in drinking water to prevent caries, discussed below, are based on a sub-mottling dosage it is difficult to understand how objection can be raised on the basis of injury to the skeleton.

6. *Practical Applications of Fluorides Through Water Supplies*

Cox, Matuschak, Dixon, Dodds, and Walker (57) discussed the addition of fluorides to water for the prevention of dental caries on a basis of an optimum intake. They proposed adjustment of the concentration to compensate particularly for seasonal and climatic variation. Cox (53) further discussed the fluoridization of water supplies as one certain method of effecting mass prevention of dental caries. He reiterated the advisability of season and climatic variation of dosage to achieve the best results with the minimum risk.

Arnold (14) reviewed the evidence of a lowered rate of caries from fluorides in water and analyzed the possibilities of danger of injury. He properly limited his consideration of injury to manifestation of mottled enamel instead of including osteosclerosis which appears at a higher level of fluorine intake. He considered the comments "that teeth in endemic areas that become carious are lost early in life because caries progresses rapidly" (188) were "without the support of controlled scientific evidence" and that the difficulty of making restorations does not enter in the dental

conditions observed at a level of 1 p.p.m. of fluorine in the water. Arnold said in conclusion: "The addition of small amounts of fluorides (about 1 p.p.m.) to fluoride-free public water supplies for the purpose of partially controlling dental caries is strongly suggested on the basis of epidemiologic and experimental evidence."

Pursuit of such a recommendation as that of Arnold would undoubtedly be safe but the results achieved could not be better and probably would be worse than those to be obtained by following the suggestion of Cox(53) that the fluoride addition be made with *seasonal* variation in accordance with the amount of water consumed. *Climatic* variation, that is regional differences in temperature, are considered by Arnold but not the difference between summer and winter.

Ast (16) reviewed the literature of fluorine in mottled enamel and caries etiology and also the toxicology of that element. He proposed the addition of sodium fluoride to water to raise its fluorine content to 0.8 p.p.m. and to observe the effect on caries as compared with a control community with 0.1 p.p.m. or less fluorine. The study period proposed was 10 to 12 years. This latter is in conformity with the idea that fluorine acts to form caries-resistant teeth, but in introductory remarks Ast said: "Daily protection would be afforded without the public being aware of it." As he proposed a constant addition to the water supply rather than a supply regulated to the needs of the subject he indicates adherence to the posteruptive theory of benefit from fluorine. If there is no effective posteruptive action of fluorine from levels of the order of 1 p.p.m. in the drinking water, the false belief by adults that they may derive benefit by fluoridization of water may react to the detriment of this public health method.

It may be pointed out that the method proposed by Cox, Matuschak, Dixon, Dodds, and Walker is based on resistance by the tooth derived from a constant daily supply of fluorine during formation of the teeth. The method of Arnold and of Ast derives from the local environment theory and so proposes a constant level of fluorides in water as it enters the mouth.

7. The Opposition to Fluoridization of Water Supplies

Specific examination of the opposition to the use of fluorides in water supplies for the prevention of dental caries will be restricted here to the analysis of a single typical paper. Smith and Smith (188) contributed a paper in September, 1940, that has since been the mainstay of those who have expressed dissent even to consideration of such an idea.

Smith and Smith (188) cited Dean, *et al.* (69) as having reported "that the incidence of caries was appreciably less in the mottled teeth..." Dean, *et al.* (69) in this, as well as in subsequent papers, pointed out that freedom from caries was shown by *teeth that were not mottled*. They (69)

said of Galesburg: "It would appear that the factor responsible for the low amount of caries in this city was operative irrespective of whether the child showed macroscopic evidence of mottled enamel." Dean and his coworkers did hesitate to "conclude that the fluorine content of the water was the only factor" but they did not say anything in the text about calcium and magnesium. These elements were only shown in their Table XIII which is a report of water analysis.

In the work of Cox, *et al.* (57) cited by Smith and Smith (188) there is no mention of mottled enamel, and Cox (1) and Cox and Levin (6) have since endeavored to correct this statement of the Smiths by pointing out that the teeth were not mottled. Cox, Matuschak, Dixon, and Walker (58) and Dixon and Cox (73) were the first to produce and observe mottled enamel in rat molars, the publications being separate from Cox, *et al.* (57). In fact, mottling of rat molar enamel was offered as a tool for the study of the relation of mottled enamel to caries (58).

The "rather sweeping conclusions" made by Cox, *et al.* were based on the (a) analysis of enamel for fluorine by Armstrong and Brekhuis; (b) the field studies by Dean, *et al.*; and (c) the experimental findings of Cox, *et al.* This chain of evidence is closely similar to that used by the Smiths to extend, in the case of mottled enamel, their "findings made on rats to the dental ills of the human race," namely, chemical analysis, field and clinical studies, and experimentation on the rat. The "mass reduction of dental caries" is parallel to their idea of prevention of mottled enamel through water supplies, the difference being that in one case the water carries an excess of a substance and in the other a deficit. The coincidence that it is the same element, excessive or deficient, has been a difficult obstacle to understanding by many others than the Smiths.

The Smiths asserted that mottled enamel "affects the teeth of every person who drinks water containing as little as 1 p.p.m. of fluorine during the years of tooth formation." This may be true in Arizona, where temperatures are high, but it does not apply in Galesburg, Illinois, where Dean, *et al.* (69), in the paper cited by the Smiths, reported that 70 of 243 of the children examined had no detectable trace of mottled enamel. This error of the Smiths accentuates that it is not the level of fluorine in the water that causes mottled enamel or gives the best protection to teeth, but the amount of fluorine ingested. This amount of fluorine varies principally with those factors that alter water intake, such as activity and seasonal, climatic, and artificial variation of environmental temperature. It is for protection from both mottled enamel and dental caries that "means of control of fluorine in the whole dietary of children should be undertaken."

The evidence presented by the Smiths that mottled teeth become carious in the older groups is in the form of a chart showing percentages with no

statement of the number of subjects involved. Inspection of the chart shows a sufficient number of the graphs to be of equal height to suggest that the numbers in each group were small. Search for ratios to fit the heights yields numbers of the order of 10 to 16 or multiples of the same. The size of the population as a whole suggests that the groups could not have exceeded 20 to 32. This chart, repeated in "Dental Caries" (1), is an example of how original data can be lost by presentation of ratios. The graphical representation does nothing to retrieve the loss.

The Smiths have not made any statement as to the number of artificial dentures elected for esthetic reasons; their description of dental caries consists of the assertion that caries existed. There is no information given as to which teeth were affected or how many.

The Smiths assert that "the range between toxic and non-toxic levels of fluorine ingestion is very small." They gave no figures and none are available on any precise basis. The absolute intake of fluorine from all sources, determined with that from water held constant with temperature fluctuation, must be found in order to indicate the threshold of toxicity. The difference between the optimum for dental caries protection and the minimum for toxic effect may be found to be small. If it amounts to the difference of adding 1 ton of sodium fluoride to 100,000,000 gal. of water to produce optimum effects, or 2 tons to produce the minimum of toxic symptoms, one would expect that this degree of precision could easily be obtained by those engaged in water treatment. Furthermore, with chemical analysis of the water as a control and, if deemed necessary, biological test, it would seem that the hazard of adding fluorides to water is nil.

Wilson (207) has noticed the paper of Smith and Smith (188). She found: "Among 90 rural housewives in Oxfordshire, aged 21-45 years, 19 had false teeth; 13 who showed mottled enamel varying from F2 to F7 on Dean's scale had but small amount of caries; while the remaining 58 gave no evidence of dental fluorosis but showed a considerable amount of decay." Wilson's examination of 1048 children revealed a highly significant negative association of dental caries with dental fluorosis, incidentally, her frequencies being compared with the χ^2 distribution. Arnold (14) has marshalled some citations against the assertion of early loss of mottled teeth by caries. As he points out, the mottling of teeth does not enter into the problem at levels of 1 p.p.m., his recommended addition of fluorides. The evaluation by the Smiths of fluorine in the prevention of caries has reached the stage of perpetuation in the textbooks (178).

VIII. GENERAL CONCLUSIONS

1. A working hypothesis that each cavity arises as a result of a unique series of events is proposed to replace the current hypothesis that every

cavity is the result of an identical series of causes or even from a single cause. The necessary revisions of procedures have been discussed.

2. Caries indices and systems of recording of dental caries have been criticized as concealing data. A system based on empirical sites of decay and maintenance of separation of data has been proposed.

3. Coarse cereal caries in rats may serve in the study of many phases of dental caries as a guide to the study of caries in man.

4. It is suggested on the basis of dental caries in rats that fermentable carbohydrates do not *initiate* caries although they do promote the enlargement of cavities by sustaining the activities of acidogenic organisms. This difference of action of carbohydrates is evidence that the mechanism of initiation of caries is distinct from that of cavity development.

5. Structure of the enamel should be considered as an important factor in determining whether or not caries will be established.

6. Acidogenic bacteria account for demineralization of enamel and dentin in caries but their relation to initiation of caries remains in the nature of association rather than proved causation.

7. Various organisms are active in caries either as a succession of types or in symbiosis or by both forms of association.

8. The study of the complete flora of the mouth, and particularly of plaques, is more promising of clarifying the local environment phase of caries than concentration of attention on a single type of organism.

9. Preeruptive fluoride is one type of mineral nutrition that results in decrease in caries susceptibility.

10. The use of fluorides in the water supplies of communities appears to be a safe practical way to prevent much tooth decay, particularly in the anterior teeth.

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Vitamins and Cancer

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I. INTRODUCTION

The view that diet may be an important factor influencing the genesis and growth of neoplastic tissues has been advanced many times in past and present-day cancer research. With the experimental recognition of various accessory growth factors or vitamins, more and more investigators have attempted to influence and control experimental and clinical cancer by means of vitamin deficiencies or excesses. The rationale of many of these efforts has been based on the consideration that malignant tissues, which are frequently characterized by a relatively rapid rate of growth, might have some excessive requirements for vitamins, and thus be differentially influenced by their omission or supplementation in the diet. At the present time, concrete evidence showing peculiar or specific relationships between vitamins and malignant growth is rather generally lacking. Advances in the science of nutrition, including the availability of synthetic diets and inbred strains of animals, and the isolation and large scale manufacture of many of the vitamins, are now providing, however, the necessary background and material for experiments which should elucidate the rôle of vitamins in the genesis and growth of malignant tissue.

The functions of vitamins in normal and malignant growth will undoubtedly be understood one day in terms of the rôles of the vitamins in the biochemistry and physiology of the tissues themselves. Interesting developments in this direction have already demonstrated that certain vitamins are the prosthetic groups of enzymes. As such they are able, even in the minute quantities in which they occur in tissues, to exert a profound controlling influence on the growth and metabolism of cells and tissues. Although only a number of the vitamins have yet been shown to participate as enzymatic prosthetic groups, or in other equivalent connections with enzymatic action, it seems not unlikely that such rôles may eventually be assigned to practically all vitamins.

It will be the function of this review to summarize the diverse information now available on the relation of vitamins to neoplasms, to bring out certain experimental complications inherent in dietary cancer research, and to consider some profitable lines for future investigation. Studies on the relations between vitamins and malignant growth have, with few exceptions, not passed the descriptive stage, and therefore this report must of necessity consist first and foremost of a collection of reported experimental observations. Although it is tempting to draw generalizations on the basis of certain experimentation, it will become evident in the following pages that most of the observations are not capable of being fitted into a unified and consistent pattern. The literature on vitamins and cancer is very scattered, and to our knowledge there is no previous report where the great

bulk of it has been brought together within recent years (see, however, 225, 114, and 118). Thus, in the 1939 monograph "Vitamins" (10a) issued by the American Medical Association, there were only some 20 references all told, and these with respect to only four vitamins (A, B₂, C, and E); in last year's "Vitamins and Hormones" (Vol. I) there were three minor references to vitamins and cancer, on pages 165, 246, and 295, in addition to a brief discussion on choline and azo dye-induced hepatomas, pp. 38-40; and similarly with respect to recent volumes of the Annual Review of Biochemistry. The new, useful book by Stern and Willheim (225) gives over a hundred recent experimental references. We have necessarily felt it our first duty to gather together here at least 80-95 percent of the available literature. We have, however, restricted the review almost entirely to observations made during the last decade, starting with the period since which vitamins have been effectively isolated, identified, and synthesized for experimental use. This has also been desirable because of the difficulty of interpreting much of the earlier work in view of the uncertainty regarding the vitamin sources. For discussion of this earlier work on vitamins and cancer the reader may be referred to several older reviews (56, 57, 77, 114, 118, 225).

In view of the present early stage of development of vitamin-cancer research, it has seemed desirable still to postpone any predominantly critical and detailed account of selected literature and, for several reasons, to avoid any attitude of sitting in final judgment, indicating settled disfavor or favor. We are aware of no paper in the field about which several to a dozen pertinent criticisms of commission or omission could not be cited, with respect to: need for ascertaining actual certainty of existence of alleged vitamin deficiencies; calorie intake regulation; use of purer vitamins, more appropriate vitamin levels, and more highly synthetic diets; unrecognized existence of multiple deficiencies; vitamin analyses of basal diet and pertinent tissues or body fluids; rôle of intestinal synthesis or destruction, absorption, detoxification, and caprophagy (principles of nutrition generally); need for more animals and purer stocks; extension of results to other types of tumors and (homologous or non-homologous) tissues; improved histopathological control; consideration of incidence in terms of both induction (latent) phase and final (long-time) period; distinction between tumor initiation and tumor growth (causal and formal genesis); etc. Obviously it would not be possible in a review of this length to criticize any appreciable number of papers in the light of even a majority of these items. Such criticism could, in our opinion, be leveled so generally and uniformly that it does not seem fair to either previous workers or present readers to make at this stage any very definite

commitment and assortment as to real and established worth of the papers in the literature, except possibly on a basis of recency or extent of work involved.

It is our opinion, therefore, that the data reported upon should, almost without exception, be regarded with the scientist's greatest weapon, attribute, and privilege, namely, suspended judgment, and, we would also plead, with considerable tolerance. Surprising as it may seem, it is not the papers in which positive claims, eventually shown to be incorrect, were made, that will have done the most harm in the long run, but it will be those papers in which negative results were reported where positive ones were inherently warranted. It is not the early paper in which a positive effect was obtained with a crude vitamin concentrate that will have been the most misleading, but the paper where multiple deficiencies existed and resulted in hiding potentially correct positive results with respect to any one deficiency. An excellent illustration of the latter instance is found in the report made five years ago (175, 177) to the effect that the marked protective effect of liver or yeast against induced hepatoma formation was not given by riboflavin or protein alone or in combination; we now know that marked protection is, in fact, given by riboflavin and protein in combination, but at considerably higher dosages than were originally employed.

In view of the foregoing comments, our chief aim here has been to give an idea of approximately what constitutes the reported research and general scope of the field of vitamin-cancer research at present, rather than narrowly to review restricted aspects with a high degree of criticism that would not seem as useful or warranted at this time. We have attempted to state the essence and claim of a given piece of research, and we have, furthermore, rather deliberately avoided mentioning the levels of vitamins employed, other dietary components, numbers of animals involved, duration of experiment, and many similar important and defining details that the interested reader must refer back to in the original literature for an adequate idea of the setting of particular experiments. To avoid needless repetition of qualification throughout the report, it is to be understood that all work mentioned, without exception, is obviously subject to confirmation, extension, or rejection. No idea of finality or completeness will be intended anywhere, regardless of any possibly unguarded implication that may appear to such effect. It is in the nature of vitamin research, where the results of ultimate interest are expressed largely in terms of biological responses, gross or microscopic, obvious or subtle, to be less definable and less generally reproducible, and this becomes all the more true when the variables of the cancer problem are added for further consideration.

II. THE RELATION OF INDIVIDUAL VITAMINS TO MALIGNANT GROWTH

1. *Vitamin A*

The well-known work of Wald and others has indicated that vitamin A functions as a prosthetic group of a retinal enzyme, visual purple. Its possible function as a prosthetic group of other enzymes has not been demonstrated as yet. Wolbach and Howe (262) and others have been impressed with the hyperplasia in a number of epithelial tissues of rats on a vitamin-A-deficient diet. McCullough and Dalldorf (150) found that a vitamin A deficiency was the primary essential condition for the production of epithelial metaplasia by mechanical irritation or theelin in rats. The function of vitamin A in controlling cell proliferation obviously gives it special interest in cancer vitamin research.

a) *The Vitamin A Content of Tumors.* Not many studies on the vitamin A content of tumor tissues have been made, probably because most tissues contain barely demonstrable amounts of it. The vitamin A content of horse melanoma and muscle was studied by Gudjonsson and Oppenheim (107) by a rat feeding assay method. They found little or no vitamin A in either of these tissues. Vogt (249, 250) found vitamin A almost exclusively in the liver and in fat. A little vitamin A was found in several tumors and there appeared to be some relationship between the vitamin A content and the malignancy of the tumors, the more malignant tumors containing somewhat more vitamin A. Sumi and Nakahara (232) found no vitamin A in Rous chicken sarcoma, Fujiwara rat sarcoma, Flexner-Jobling rat carcinoma or Bashford mouse carcinoma.

More recently Seeger (208), using histochemical staining methods, reported that the vitamin A content of exudate cells from Erlich mouse sarcoma ascites varied with the "degree of malignancy". Of the four types of exudate cells found in these ascites, the one designated as precancerous showed somewhat more vitamin A than normals. The more cancerous exudate cells contained much less vitamin A than normal exudate cells. On the whole the results indicated that in this particular series the more malignant exudate cells tended to contain less vitamin A than normal exudate cells.

Popper and Ragins (191) using a histofluorescence technique, showed that the vitamin A content of tumor tissues was largely dependent upon the vitamin A content of the tissue of origin. Vitamin A was found only in those tumors which arose from tissues containing vitamin A. Usually the tumor contained somewhat less vitamin A than the tissue of origin. The distribution of vitamin A in tumors suggested that the presence or absence of the vitamin does not influence tumor formation. Not all tumors derived from tissues containing vitamin A contain measurable

quantities of it. Goerner and Goerner (99) found that the tumors arising in the livers of rats fed 2-amino-5-azotoluene contained no vitamin A, whereas liver surrounding the tumors contained about 100 U.S.P. Units per g. of tissue, and normal liver contained about 400 Units per gram.

b) *Vitamin A and Gastric Cancer.* A more extended discussion of the possible relationship between dietary vitamin deficiencies and gastrointestinal lesions will be given later. It is clearly established that benign hyperplasia and papillomatosis may arise in rats deficient in vitamin A. However, as has already been pointed out, such lesions may be produced in animals on diets deficient in factors other than vitamin A, and there is no reason to believe that the protective action of vitamin A is a particularly specific one. Also evidence is lacking that the hypertrophic lesions bear any relation to gastric carcinoma since the lesions of rats on a low vitamin A diet disappear after the rats are returned to an adequate diet (42, 90). The demonstration of truly malignant tumors in the stomachs of animals deficient in vitamin A (or other dietary factor) would do much toward establishing the importance of diet in gastric cancer.

c) *The Effect of Carcinogens upon the Vitamin A Content of Liver.* Goerner (95) demonstrated a peculiar effect of certain carcinogens upon the vitamin A content of liver mitochondria. He found that repeated intraperitoneal injection of 1,2,5,6-dibenzanthracene markedly reduced the vitamin A content of the mitochondria isolated from the liver of rabbits. Goerner and Goerner (96) found that the mitochondria of transplanted rat tumors contained no vitamin A. Primary liver tumors induced in rats by feeding 2-amino-5-azotoluene also contained no vitamin A (99). These workers found that the stores of vitamin A in rat and rabbit liver were reduced whereas the contents of vitamins C and D were unchanged by repeated dibenzanthracene injection (97, 98). Baumann, Foster, and Lavik (22) placed rats on low vitamin A diets and found that injection of various carcinogens, especially dibenzanthracene, increased the rate of depletion of the hepatic vitamin A stores. In contrast to the results of Goerner and Goerner (99) using aminoazotoluene, Baumann, *et al.* found that *p*-dimethylaminoazobenzene caused no reduction in hepatic vitamin A. Carruthers (54) found that methylcholanthrene and benzpyrene injected intraperitoneally in large doses into rats appeared to cause some depletion of the vitamin A content of the liver. The vitamin A content of livers of mice was not nearly so affected by carcinogens as rat livers. Carruthers (54) emphasized that the use of more than one species is necessary in this work. Marron (156) showed that the hepatic vitamin A level of rats was lowered by injection of methylcholanthrene but the ability to store vitamin A was regained within 120 days after the last injection. Abels, *et al.* (2) found that the decrease in hepatic vitamin A in rats given

dibenzanthracene did not seem to be due to a general hepatic insufficiency, since many liver function tests on such animals were normal. The loss in vitamin A from the liver was thought to be a failure of the liver to store the vitamin through some specific effect of dibenzanthracene on some substance in the liver to which vitamin A might be bound.

It is difficult at the present time to evaluate the significance of the changes in vitamin A stores following administration of carcinogens, or to tell what relation this work has to the abnormal metabolism of vitamin A in cancer patients already discussed. Further work on vitamin A metabolism in relation to cancer and carcinogenesis is most certainly indicated.

d) *Vitamin A and Tumor Incidence and Growth.* Attempts to influence the incidence of various types of experimental tumors by changing the vitamin A content of the diet have been made by several groups of investigators. The incidence of spontaneous tumors in rats was not increased by their prolonged maintenance on a vitamin A free diet (Sugiura and Benedict, 229) but the extent of A deficiency obtained is uncertain, as in so many vitamin A studies. Orten, Burn, and Smith (182) found that odontoma may arise in rats fed a barely maintenance level of vitamin A for a long period of time. Davidson (61) found a somewhat higher incidence of tar induced cancer in mice on a low vitamin A diet than on a vitamin-A-supplemented diet. Howe, Elliott, and Shear (120), however, noted no significant influence of the dietary vitamin A level on the production of tumors in mice by 3,4-benzpyrene implanted subcutaneously. Bonser (33) found that deprivation of vitamin A neither stimulated nor inhibited the production of mammary tumors brought about by injection of estrin into male mice. Andervont (11) reported that the spontaneous adenomatous lesions in the pyloric stomach of strain I mice were not influenced by the dietary vitamin A level.

Contradictory results have been obtained on the effect of insufficient or excess dietary vitamin A on the growth of transplanted tumors of various kinds. Roffo (197) found that transplanted sarcomas and carcinomas grew more slowly in vitamin-A-deficient rats than in controls. Huzita (122) reported that a vitamin A deficiency inhibited, and a moderate excess stimulated the growth of a transplanted rat sarcoma. A great excess of vitamin A inhibited the growth of the tumor. The effect of vitamin A deficiency or surfeit on the radiosensitivity of the tumors paralleled the effects on growth rate (122). Kuh (141) found that a moderate supplement of vitamin A had no influence on the growth of the Twort mouse carcinoma. Very high levels of vitamin A were inhibitory to the growth of this tumor. Sure, Buchanan, and Thatcher (233) obtained no influence with massive doses of vitamin A on the growth or regression of Walker rat carcinosarcoma

256. Ihara (123) found that a deficiency of vitamin A inhibited and an excess stimulated the growth of the Flexner-Jobling rat carcinoma. Dittmar (69) found the percentage takes and the growth rate of Erlich mouse carcinoma was not influenced by addition of vitamin A to a deficient diet unless the diet was deficient for some time prior to the implantation of the tumor. Wyard (267) attempted without success to control clinical cancer by placing patients on diets low in vitamin A. Lustig and Wachtel (148) treated patients with epitheliomas by topical and subcutaneous injections of vitamin A. Some general local improvement was usually noted, and ulcers appeared to heal more satisfactorily.

Gordonoff and Ludwig (100) grew chick fibroblasts and mouse carcinoma in tissue cultures containing the plasma of rats fed on low vitamin diets. Plasma from rats on a diet supplemented with vitamin A seemingly brought about more growth of the tissue cultures than plasma from A-deficient rats. Vollmar (251) found that small amounts of vitamin A stimulated the growth of chick heart and mouse carcinoma tissues in tissue culture.

Taken as a whole, the relationship between vitamin A and malignant growth appears to be a rather nebulous one. Conclusive evidence that vitamin A is of special importance, even to a particular type of cancer, is still lacking. However, sufficient evidence has accumulated which makes it desirable to seek further for a peculiar and perhaps specific relationship between vitamin A and malignant growth. This evidence consists of the demonstration of hyperplastic lesions of epithelial linings in vitamin A deficiency, possible alteration of vitamin A metabolism in tumor-bearing patients, the effect of carcinogens on vitamin A metabolism, and occasional experiments indicating that the dietary vitamin A level may influence the growth and incidence of experimental cancer.

2. B Complex Vitamins

A great deal of research has been carried out on the effects of yeast and liver on the incidence and growth of various types of cancer. These materials are rich sources of vitamins of the B complex, and their influence on malignant growth has often been attributed to this high B complex content. Some of the more recent papers in which yeast and liver have been shown to influence production of azo dye hepatomas and dietary gastro-intestinal lesions will be discussed later. However, it is recognized that other vitamin or non-vitamin (protein) components of these crude materials may have important effects on cancer incidence and growth, and so subsequent discussion will be limited to those B complex vitamins which have been tested in pure or relatively pure form.

a) *Biotin*. Biotin, one of the last of the B complex vitamins to have its chemical structure elucidated, has not as yet been identified as part of any

specific enzyme system. Biotin has, however, already been linked rather closely with the development of cancer. Originally back of such work was the compelling incentive for experimentation derived from the thought that biotin, by virtue of its identity with coenzyme R, is a respiratory catalyst (10), and might thus in some manner protect against tumor formation by acting to overcome the long spoken-of "defect" in tumor respiration, variously described and interpreted by Warburg and Dickens over a decade ago, and more recently by Potter, Stotz, Baumann, Burk, and others. If biotin were to overcome the defect in tumor respiration, it might thereby reduce the high fermentation so characteristic of tumors (including butter yellow tumors, 44, 45), and thus perhaps in turn prevent tumor formation or development. However, biotin also catalyzes the fermentation of yeast even more markedly and directly than the respiration (50), so that it is also conceivable that biotin might indeed promote rather than protect against tumor formation, that is, be procarcinogenic rather than anti-carcinogenic.

The observation that pure biotin can, in fact, overcome the protective action of a riboflavin-casein-vitamin supplemented diet in the formation of hepatomas by *p*-dimethylaminoazobenzene feeding and the protective action of avidin (egg white) feeding against such tumors (245, 47, 160, 221) will be discussed later. This procarcinogenic effect of biotin has not been adequately tested with other types of tumor, and conclusions as to the general nature of the observations with butter yellow tumors must be held in reserve.

In this connection, however, Thompson and Voegtlin (242), at this institute, have noted in a proportion of rats an apparent growth promoting action of both excess biotin and riboflavin added either singly or in combination, upon actively regressing hepatoma 31. Their animals were all on a semisynthetic casein diet supplemented daily with 400 mg. of dried yeast. The biotin and riboflavin were used in daily supplement in the following effective dosages:

10 or 20 γ biotin.

10 or 20 γ biotin plus 400 γ riboflavin

200 to 3200 γ riboflavin

10 or 20 γ biotin injected subcutaneously

10 or 20 γ biotin injected subcutaneously plus 4 mg. riboflavin

From the data at present available, it can be said that the biotin-riboflavin combination was more effective than riboflavin alone, while (in the absence of excess riboflavin supplement) biotin injected subcutaneously was apparently less effective than the same amount ingested.

West and Woglom (255, 256) reported that a number of tumor tissues contained more biotin than the homologous normal tissues. The liver was

an exception since hepatomas contained less biotin than normal liver. It was concluded that the biotin levels of normal and embryonic tissues deviated in the same direction from the homologous normal tissue, and West and Woglom suggested that the difference in the biotin content of normal and tumor tissue reflected differences in the metabolism of these tissues. By maintaining tumor bearing mice on egg white diets West and Woglom (256) found that the biotin content of the tumor could be markedly reduced with no great influence on the growth of the tumors.

Pollack and co-workers (188, 189) determined the biotin content of a great many neoplastic and normal tissues, and concluded that probably cancers neither require nor are associated with a high biotin content. Taylor, Pollack, and Sortomme (239) found that egg white feeding had no marked effect on the growth or percentage takes of transplanted mouse mammary tumors, although simultaneous addition of several B vitamins caused some increased resistance to implantation of this tumor.

Laurence (145) speculated that malignant cells might have a higher biotin requirement than normal cells, and that a deficiency in biotin might thus lead to a differential inhibition of malignant tissue. An attempt to test this hypothesis clinically was made by Kaplan, Zurrow, and Goldfeder (129), and some benefit was claimed in a very limited number of cases. Rhoads and Abels (193) found that there was no improvement in two cancer patients who were fed large amounts of egg white or avidin concentrates. These patients, however, showed no symptoms of biotin deficiency even after a long period on the avidin-containing diet. It is interesting to note that a patient showing clinical signs of what was very possibly a naturally occurring biotin deficiency developed a carcinoma of the penis (Williams, 260). Thus, up to the present time there is no definite evidence that supports the hypothesis that human cancer may be controlled by developing a biotin deficiency. Actually, such control might hardly be expected on the basis of available observations, which do not support the basic hypothesis that cancer tissue has markedly heightened biotin requirements.

A development that will undoubtedly complicate studies on the relation between biotin and cancer are the observations of Oppel (179) and Burk and Winzler (49) which indicate that there are several naturally occurring biotin vitamers, some of which are active in yeast or rhizobium biotin assay methods but which do not cure biotin deficiency in animals. In biotin content of tissue, and biotin balance, studies, care must be taken to use methods which will distinguish between animal-active, avidin-combinable biotin, and animal-inactive or avidin-uncombinable biotin forms. Our studies have indicated that in biotin-deficient rats, the percentage of avidin-uncombinable biotin in normal and tumor tissues rises to many

times normal values of 0.1-5 per cent, up to as high as 80-90 per cent.

b) *Riboflavin*. The participation of riboflavin as an integral part of a number of enzymes is now well established. In general these are flavoproteins with the riboflavin-containing prosthetic groups more or less dissociable from the protein. The old and new yellow enzymes, xanthine oxidase, fumaric dehydrogenase, aldehyde oxidase, *d*-amino acid oxidase, cytochrome C reductase and possibly succinic dehydrogenase, have been indicated to be flavoproteins. The protective action of riboflavin against production of hepatomas by the oral administration of *p*-dimethylaminoazobenzene is one of the most clearly established instances of the influence of a vitamin on malignant growth (18, 46, 48, 108, 135, 160 192a). However, little evidence yet has accumulated to indicate that riboflavin has protective action against other types of animal tumors, indeed, rather the contrary. The effect of riboflavin-deficient diets on the incidence and growth of spontaneous mammary tumors in adult C3H mice has been studied by Morris and Robertson (169, 172), who found that the growth rate of the tumors was greatly retarded in mice on riboflavin-deficient diets. However, the weight of the host animal commenced to decrease even before effects on tumor growth were noted, almost immediately upon removal of riboflavin from the diet. The riboflavin content of the muscles and livers of mice fed riboflavin-deficient diets fell 30 to 50 per cent, somewhat before corresponding decreases occurred in the tumors. A diet low in riboflavin also inhibited the growth of transplanted rat liver tumors (hepatoma 31). Perhaps the most significant observation was that high riboflavin levels caused after 4 weeks an abnormally large average number of spontaneous mammary tumors per adult C3H mouse, namely, 2.7 at 10 micrograms per g. of food and 2.2 at 3.3 micrograms, as compared with an average of 1.8 tumors per mouse at maintenance riboflavin levels of 1.5 micrograms and only 1.5 tumors per mouse for a highly-deficient riboflavin level of 0.5 micrograms (residual riboflavin in the diet). The last-named level produced death due to deficiency at about nine weeks (147), the food intake being little affected by any of these riboflavin levels. These striking results obviously raise the question of the possible effect of high riboflavin levels on breast tumor occurrence and metastasis in women, both before and after appearance of the first tumor. It is to be emphasized that high oral riboflavin levels (only three to seven times the maintenance level) produced more tumors per mouse than any other experimental or stock diet studied, rather than that high riboflavin deficiency caused any great reduction compared to normal or maintenance riboflavin. Interestingly enough, although the average number and size of tumors considerably increased with riboflavin level, the mice lived considerably longer, indicating that the resistance of the mice to the deleterious effects of tumors was in some

manner markedly increased by the riboflavin (all mice were on a riboflavin-deficient diet for three weeks before the riboflavin supplementations). As already mentioned, Thompson and Voegtlin (242) have observed a reversal of regression and resumption of growth by transplanted hepatoma 31 tumors in rats when excess riboflavin (0.2 to 4 mg./rat/day) was added to the semisynthetic, yeast-supplemented, casein diet of animals on which this tumor was actively regressing. Simultaneous addition of excess biotin (10 or 20 micrograms/rat/day) increased the frequency of regression reversal, while excess biotin alone without excess riboflavin was definitely effective. Cessation of regression was never observed to occur in the absence of both the biotin and riboflavin excess supplements.

Taylor, Pollack, and Sortomme (239) found that the addition of riboflavin, niacin, and pantothenic acid to a purina chow diet resulted in a definite decrease in the number of takes of transplanted mammary carcinoma in mice. Resistance to this tumor was not further increased by adding four more of the B vitamins with or without additional yeast extract. Bischoff and Long (27) found that the addition of riboflavin and thiamine to a B complex-deficient diet had no more effect on the growth of implanted mouse sarcoma 180 than on the host. Lewisohn, *et al.* (146) have reported that the injection of yeast extract reduced the percentage takes of a transplanted mouse adenocarcinoma, and that the addition of riboflavin further reduced the percentage takes. Lauber, Shock, and Bersin (144) reported that parenteral administration of riboflavin, thiamine, and ascorbic acid to mice painted with methylcholanthrene did not influence the production or growth of tumors. Henkel (118) injected riboflavin into mice bearing ascite carcinoma, and found no effect on the growth of tumors. Vollmar (251) found that addition of riboflavin to tissue cultures of normal chick fibroblasts stimulated their growth, whereas the growth of mouse tumor tissue cultures was inhibited by the same concentration of riboflavin.

Brabec (39) reported that a rat sarcoma contained much less riboflavin than liver, and that diets deficient in riboflavin did not prevent the takes or growth of transplanted rat sarcomas. The riboflavin content of rat liver and hepatoma has been studied by Masayama and Yokoyama (159), Kensler, Sugiura, and Rhoads (134), Kahler and Davis (128), and Robertson and Kahler (196), and in all cases the riboflavin content was lower in the liver tumors than in the normal liver. Robertson and Kahler (196) found that fifteen mouse and rat tumors were very similar in their riboflavin contents (20-30 γ /g. dry). Decreases in the riboflavin content as well as in flavin containing enzymes accompanying the production of *p*-dimethylaminoazobenzene hepatomas have been summarized in Table I. Pollack and co-workers (187, 188) analyzed a number of mouse, rat, and human

cancer and normal tissues for their riboflavin content and concluded that the cancers in general contained somewhat less riboflavin than most normal tissues and considerably less than those tissues especially rich in riboflavin (liver, heart, kidney).

Figge and Strong (87) reported that the xanthine oxidase activity of the livers of C3H mice was half as great as that of the J.K. strain, and this observation was correlated with the higher susceptibility of C3H mice to spontaneous and induced cancer. It was suggested that the possible function of xanthine oxidase in nucleoprotein metabolism might be responsible for the difference in the cancer susceptibility of the two strains, due to the difference in tissue xanthine oxidase.

The metabolism of tumor tissue is partially characterized by its high aerobic and anaerobic glycolysis. Since several of the flavoprotein enzymes have been shown to function in the transfer of hydrogen and electrons to molecular oxygen, it has been suggested that excess riboflavin might divert the metabolism of cancer tissues toward a more normal oxidative type. So far no direct evidence supporting this attractive hypothesis has been presented, but further investigations should be carried out.

c) *Thiamine*. Phosphate esters of thiamine participate in the prosthetic groups of cocarboxylase, and several other enzymes active in the metabolism of microorganisms and animal tissues (21). The well established fact that cocarboxylase plays a very important rôle in the metabolism of the key metabolite, pyruvic acid, means that a great many catabolic and anabolic processes are disturbed in a thiamine deficiency. A possible differential effect of a thiamine deficiency on the genesis or growth of normal and cancer tissues might possibly be expected in view of the actual differences in their metabolism.

Several attempts have been made to influence the incidence and growth of experimental tumors by changing the thiamine content of the diet. Jones (127) reported that transplants of Jensen sarcoma into rats kept on a low thiamine diet appeared later and grew more slowly than controls. Addition of thiamine to the diet of the thiamine-deficient, tumor-bearing rats frequently resulted in a temporary stimulation in both body weight and growth of tumors, followed by regression of the latter, indicating a continued effect of the earlier thiamine deprivation. Caloric intake was definitely affected by the thiamine regulation and undoubtedly influenced the results in a manner somewhat difficult to ascertain. A clearer picture is given by the work of Morris (169) in which it was found that adult C3H mice promptly lost weight on a diet greatly deficient in thiamine, due to decreased food consumption, and died in two to four weeks, whereas tumors on such animals continued to grow for about two weeks and then slightly decreased. Tumors in control animals fed the same amounts of

food containing excess thiamine responded exactly like those on the deficient diet, indicating that caloric intake rather than thiamine level was the dominating factor here.

Gordonoff and Ludwig (102) reported the dietary thiamine level did not influence the appearance or growth of methylcholanthrene induced skin tumors in mice. Maisson, Pourbaix, and Camerman (153) found that oral administration of thiamine and niacin reduced the incidence of tumors in mice painted with benzpyrene. The possibility that a thiamine deficiency might account for the lower tumor growth rate sometimes observed in hyperthyroidism was considered by Beck and Krantz (23). However, they found no consistent difference in the growth of Walker sarcoma 39 in thyroid-fed or thyroidectomized rats with or without thiamine supplementation. Bischoff and Long (27) found that the addition of thiamine and riboflavin had no effect upon the growth rate of sarcoma 180 in mice. No pronounced effect of thiamine has been observed on the production of liver tumors by *p*-dimethylaminoazobenzene feeding such as was found with biotin or riboflavin (245, 47, 242, 160). Lauber, Shock, and Bersin (144) found that the parenteral administration of thiamine along with riboflavin and ascorbic acid had no influence on the incidence of tumors in mice induced by methylcholanthrene painting.

In tissue culture Vollmar (251) observed that the growth of chick fibroblasts was stimulated by concentrations of thiamine that inhibited the growth of cultures of Ehrlich mouse carcinoma. Gordonoff and Ludwig (100, 101) found that tumor tissue cultures grew more slowly in plasma from rats fed a diet deficient in thiamine than in the plasma of normal rats.

The thiamine content of a large number of normal and tumor tissues has been studied by the Texas group (188, 240). Their data shows no consistent general difference in the thiamine content of normal and tumor tissues. Masayama and Yokoyama (159) likewise found only minor differences between the thiamine content of normal rat liver and hepatoma. Seeger (209) found thiamine in exudate cells from Ehrlich mouse ascite carcinomas.

Abnormalities in the metabolism of thiamine which appear to occur in the white cells of leukemic patients (1) will be discussed later.

It is clear that the results of present investigations of the relation between thiamine and malignant growth are inconclusive, and further work will be necessary to determine whether or not thiamine has any peculiar or special rôle in cancer as clearly distinguished from general growth.

d) Pantothenic Acid. Pantothenic acid is one of the more recent additions to the chemically identified members of the vitamin B complex, and comparatively little is known about its functions in cells. Yeast grown at low levels of pantothenic acid has a low respiration rate, an indication that the

vitamin is concerned in some oxidative reaction. Pantothenic acid is firmly bound to tissue proteins. These observations indicate that the vitamin probably functions in an enzyme system involved in metabolism.

Morris and Lippincott (170) found that a deficiency of pantothenic acid caused a marked depression in the growth rate of spontaneous mammary carcinomas in C3H mice. The tumor growth rate in pantothenic-acid-deficient animals was stimulated by the addition to the diet of pantothenic acid. There was no selective influence on the tumor since the retardation of tumor growth by pantothenic acid deficiency resulted simultaneously in severe disturbances in the nutritional status of the host. The actual incidence of tumors was not noticeably affected by pantothenic acid level, as it was so markedly in the corresponding experiments with riboflavin (169, 172) already discussed. Bischoff, Ingraham, and Rupp (26) found that pantothenic acid deficiency of relatively short duration did not markedly affect the growth of sarcoma 180 in mice.

Lewisohn, *et al.* (146) reported that pantothenic acid added to intravenously injected yeast extract decreased the percentage of takes by implanted mouse carcinoma 2163. Pantothenic acid alone, however, had no influence on the growth rate or the percentage take of the tumors.

Taylor, *et al.* (188, 238, 240) determined the pantothenic acid content of many mouse, rat, and human cancer and normal tissues. They found the pantothenic acid content of tumor tissues to be about the same as such normal tissues as spleen, lung, and muscle, but much lower than pantothenic-acid-rich tissues like liver, heart, and kidney.

Results to date on the occurrence and effect of pantothenic acid in neoplasms suggest that cancer tissue has no greater need for pantothenic acid than normal tissues.

e) Niacin. Coenzyme 1 (diphosphopyridine nucleotide) and coenzyme 2 (triphosphopyridine nucleotide) contain niacin. These two coenzymes are of utmost importance as hydrogen carriers in intermediary metabolism, both aerobic and anaerobic. Therefore it might be suspected that niacin deprivation might have a greater effect on rapidly growing and glycolyzing tumor tissue than on normal tissue. Actually no data have yet been collected which support such a hypothesis. Experiments along these lines have only just commenced.

Willheim and Bocobo (259) injected daily massive doses of niacin into patients with oral cavity carcinomas and noted an increase in keratinization of the cancer and some transitory local and general improvement. Maison, Pourbaix, and Camerman (153) found that nicotinamide delayed the formation of benzpyrene skin tumors in mice. Bernheim and Felsonvanyi (25) analyzed Walker rat carcinosarcoma 256 and other rat tissues for coenzymes 1 and 2 and found less of the coenzymes in the cancer than in the

normal tissues which they analyzed. Kensler, Sugiura, and Rhoads (134) found that the coenzyme 1 content of rat liver was lowered in rats fed *p*-dimethylaminoazobenzene and fell even lower in the hepatomas produced by this agent. Kensler, *et al.* (135) also found that the addition of niacin slightly increased the protection of riboflavin-casein diets against *p*-dimethylaminoazobenzene induced hepatomas. Antopol and Unna (18) noted no such effect of niacin with respect to liver pathology. Taylor, *et al.* (237, 188) analyzed various normal and tumor tissues for niacin. Their results indicated that the niacin content of tumor tissues, in general, was somewhat lower than that of most normal tissues.

f) *Pyridoxin*. Little is known of the function of pyridoxin in cells, and only a few experiments on the effect of pyridoxin deficiency or surfeit on malignant growth have been carried out. A procarcinogenic action of pyridoxin on hepatoma induction in rats fed *p*-dimethylaminoazobenzene has been observed by Miner, *et al.* (161).

Bischoff, Ingraham, and Rupp (26) found that the omission of pyridoxin from an otherwise adequate diet produced a marked decrease in the growth rate of sarcoma 180 in mice, and that the addition of pyridoxin to this diet corrected this defect. The addition of pyridoxin to a B-complex-deficient diet produced a significant increase in rate of tumor growth. The effects of pyridoxin deficiency or supplementation appeared to influence tumor growth more than somatic growth, a condition quite the opposite of that previously obtained with thiamine and riboflavin (27). On the other hand, Morris (169) at this institute has found that whereas net body weight (*i.e.* corrected for tumor weight) of adult C3H mice was definitely affected by pyridoxin level, neither the growth nor incidence of spontaneous mammary tumors on these mice was affected by either a pyridoxin-deficient diet maintained even until death of the animals after some seven weeks' deficiency or by large doses of pyridoxin; this was in marked contrast to corresponding experiments with riboflavin (169, 172) already discussed, where both tumor growth and body weight decreased with riboflavin deficiency, and high levels of riboflavin caused a marked increase in tumor incidence. With neither the pyridoxin (nor similarly riboflavin or pantothenic acid) deficiency experiments was the food intake a marked variable, as it was in the case with thiamine (169).

Sharpless (218, 219) found that a deficiency in pyridoxin (also deficiencies in cystine, riboflavin, or choline) would promote epithelial hyperplasia and ulceration in the fore-stomachs of rats. The gastric lesions were thought to be due to the abnormal sensitivity of the epithelium due to the deficiency. The Texas group (188, 240) determined the pyridoxin content of many normal and tumor tissues, and found that the pyridoxin content of cancer tissue was, in general, somewhat lower than most normal tissues.

g) *Folic acid*. Little research has been carried out on the relation of folic acid to malignant growth. This is attributable to the relative recency of discovery of this vitamin, the absence of pure preparations, and the fact that deficiencies in animals have been obtained only by feeding them diets containing sulfaguanidine or sulfasuccidine. Taylor, Pollack, and Sortomme (239) found that addition of niacin, riboflavin, and pantothenic acid to a purina dog chow diet increased the resistance of mice to implantation of a transplanted mammary carcinoma. Further addition of folic acid along with thiamine, inositol, and pyridoxin did not further increase the resistance to the implantation of this tumor. A very interesting and suggestive observation was brought out by Pollack, Taylor, and Williams (188), who found that of the B complex vitamins in normal and cancerous tissues, folic acid was consistently found at the highest relative level in cancer tissue.

It would appear that in order to study the relation of folic acid to the incidence and growth of cancer, it will probably be necessary to employ diets including sulfasuccidine or other equivalent bacteriostatic agents in order to prevent the intestinal synthesis of this vitamin.

h) *Inositol*. A procarcinogenic effect of inositol on the production of hepatomas induced by oral administration of *p*-dimethylaminoazobenzene to rats has been noted, and the inositol content of livers and hepatomas measured (46, 160) (Table I). Taylor, Pollack, and Sortomme (239) upon adding inositol to a purina chow diet containing supplementary B vitamins, obtained no effect on the percentage of takes of a transplanted mammary carcinoma in mice. Studies of the inositol content of many normal and cancer tissues have been carried out by the Texas group (188, 240). In general the inositol content of cancer tissues was relatively high. Intravenous injections of inositol into mice bearing sarcoma 180 have been reported to inhibit the growth of this tumor (Laszlo and Leuchtenberger, 143).

i) *Choline*. As will be indicated more fully later, choline has been shown to influence both procarcinogenically and anticarcinogenically the production of hepatomas in rats by feeding *p*-dimethylaminoazobenzene (46, 72, 108, 160, 258). Choline has also been mentioned as one of the nutritional factors important in preventing the development of papillomatous hyperplasia and ulceration in the forestomachs of rats (216, 217, 218, 219). Vincent, Daum, and Bouchet (246) found considerable choline and acetyl choline in human nervous system tumors. Jacobi and Baumann (126) observed that the deficiency or excess of choline failed to alter the course of methylcholanthrene-induced warts in mice or transplanted Flexner-Jobling carcinomas in rats.

j) *p-Aminobenzoic Acid*. Both a pro- and an anti-carcinogenic action of

p-aminobenzoic acid on the production of hepatomas in rats fed *p*-dimethylaminoazobenzene has already been obtained (46, 47). Thompson and Voegtlin (242) of this institute have observed no effect on the growth of transplanted hepatoma 31 over a period of 60 days when *p*-aminobenzoic acid was administered at levels of 10, 50, 100, or 200 mg. per rat per day; a questionable effect on body weight was noted. No other information is available relating this vitamin to malignant growth.

k) *B Vitamin Uniformities*. Pollack, Taylor, and Williams (188, 241), in discussing the occurrence of B complex vitamins in normal and cancerous tissue, developed a mathematical expression for similarities or differences in the vitamin content of a series of tissues. This expression, a measure of the vitamin uniformity in different tissues, is given by 100% minus the coefficient of variation where *c.v.* is the standard deviation of the content of a particular vitamin divided by the mean. The sum of the individual vitamin uniformities divided by the number of vitamins gives the total vitamin uniformity of the series of tissues. Using this method of expression, the Texas group reported that the same type of tissue from different species had a high degree of B vitamin uniformity, while tissues of different structure and function had very low vitamin uniformity. Tumors, regardless of tissue origin, manner of induction, or host species, had a relatively high vitamin B uniformity. This could be interpreted as a basis for the thought that malignant neoplasms tend to have a similar cellular metabolism, and form, in effect, a somewhat common biochemical tissue type.

3. Vitamin C

Vitamin C has not been observed to act as a prosthetic group, but its potential connection with enzyme action is well established. A controlling action on many of the hydrolytic enzymes has been demonstrated. In view of the reversible oxidation-reduction of ascorbic acid, it has been suggested that it may function as a hydrogen transfer system or as a regulator of cellular oxidation-reduction potential.

a) *Ascorbic Acid Content of Tumors*. Boyland (35) and others (254, 265) first considered the material in normal and tumor tissue which reduced phenol 2,6-dichlorophenolindophenol to be ascorbic acid. Biological assay with guinea pigs indicated that the antiscorbutic activity and reducing power ran roughly parallel (173), although Harris (115, 116) had not obtained such close agreement. Other workers have indicated that the reducing capacity of tumors is largely due to ascorbic acid (130, 173, 247). The tissue and tumor ascorbic acid is low in guinea pigs on a scorbutic diet, and rapidly rises to normal within a short time after administration of ascorbic acid to scorbutic animals (36, 253).

The ascorbic acid content of hepatomas induced by feeding *p*-dimethyl-

aminoazobenzene and *o*-aminoazotoluene to rats may be somewhat higher, and transplanted hepatomas somewhat lower, than the normal liver (92, 124, 158, 195). Injection of the carcinogen 3,4,5,6-dibenzcarbazole into rats did not affect the vitamin C content of livers although the glutathione content was increased (Boyland and Mawson, 38). Carruthers and Suntzeff (55) observed that the ascorbic acid content of methylcholanthrene induced skin tumors in mice was lower than normal skin on a dry weight basis, but about the same as normal skin on a nucleoprotein phosphorus basis. The abnormally high ascorbic acid content of the white cells of leukemia patients found by Butler and Cushman (51) has already been mentioned.

On the whole, the ascorbic acid content of tumors appears to show no consistent tendency to be either higher or lower than the corresponding tissues of origin. This is especially clear in the work of Robertson (195) of this institute who analyzed a number of rat and mouse tumors and the tissues whence they came. In about one-half of the twenty two tissue pairs the tumors were higher; in a third the tumors were lower; and in the remaining sixth the tumors were the same as the parent tissue in their content of ascorbic acid. Various tumors ranged in content from 15 to 70 mg. ascorbic acid per 100 g. fresh weight, normal tissues from 4 to over 150 mg. A grouping of the tumors into sarcomas and carcinomas showed no difference in average ascorbic acid content or distribution range of values. Necrotic tumors contained somewhat less than healthy tumors, in harmony with observations of others (73, 265). No relation between content and rate of tumor growth was noted. Spontaneous mammary tumors in mice appeared to contain slightly less ascorbic acid in old rather than young mice, but no difference was noted in sarcoma 180 transplants on 2 months' old and 2 years' old mice, nor in leukemic nodes of young and old mice with leukosis P1534.

b) Ascorbic Acid and Tumor Growth. Pollia (190), Woodward (265), and Stepp and Schroeder (224) found that the administration of ascorbic acid to tumor-bearing rats or mice had no effect on the rate of growth of the tumors, although Woodhouse (263) observed a stimulation of the growth of tar produced neoplasms in mice. Lauber, Shock and Bersin (144) found that injection of ascorbic acid had no influence on the incidence or growth of tumors in mice painted with methylcholanthrene. Fodor and Kunos (89) found a stimulation of the growth of Ehrlich mouse carcinoma transplants when ascorbic acid was administered. Brunschwig (41) reported that an excess of ascorbic acid slightly stimulated the growth of certain transplanted rat and mouse tumors in some experiments.

In tissue culture Vogelaar and Erlichman (248) found that ascorbic acid stimulated the growth of explants of Crocker mouse sarcoma. Vollmar

(251) observed that vitamin C added to tissue cultures stimulated the growth of chick heart fibroblasts, but had no influence on mouse carcinoma cultures. Gordonoff and Ludwig (100, 101) found no difference in the action of plasma from rats fed low or high vitamin C diets on the growth of tumor tissue *in vitro*.

A criticism that can be made of most of the work on the effect of ascorbic acid on tumor growth is that the experiments have been carried out with animals that do not require ascorbic acid in the diet. Experiments on the effect of ascorbic acid on the incidence and growth of various types of tumors in the guinea pig should certainly be carried out.

c) *Other Relationships Between Ascorbic Acid and Malignant Growth.* Harde and Kobozieff (113) and Boyland and Boyland (37) found that the production of hemorrhage in transplanted mouse tumors by bacterial toxins was accompanied by a marked reduction in the ascorbic acid content of the tumors. Andervont and Shimkin (12) followed this with the demonstration that ascorbic acid prevented the hemorrhage and regression produced in transplanted mouse tumors by crude bacterial filtrates of *B. prodigiosus*. Later work has not confirmed this observation for purified polysaccharide fractions (220).

The oxidation of several carcinogenic hydrocarbons in water-acetone solution has been shown to be brought about by ascorbic acid (Warren, 252). Among the products formed during the ascorbic acid oxidation of 3,4-benzpyrene was a phenolic compound having a green fluorescence similar to the phenol isolated in urine as a metabolite of benzpyrene.

Ascorbic acid also catalyzes the oxidation of phospholipids emulsified in slightly acid solution. Many carcinogenic and related compounds were able to inhibit the catalytic oxidation of phospholipids by ascorbic acid and other agents (67, 202).

Work indicating that cancer patients may have an abnormal metabolism of ascorbic acid has already been reviewed (17, 93, 162, 223, 224).

4. Vitamin D

No general enzymatic function of vitamin D in tissues has been demonstrated, although its relation to calcium and phosphorus metabolism is well established. Vitamin D administration and deprivation is known to influence the concentration of the serum phosphatases as well as the level of tissue respiration. Relatively little research has been carried out on the relation of vitamin D to malignant growth.

Pappenheimer and Larimore (184, 185) found hyperplastic gastric lesions in rats fed a diet deficient in fat soluble vitamins, and found that cod liver oil, a rich source of vitamin D, would not prevent the development of these lesions, although it offered noticeable improvement on a certain stock diet

deficient in vitamin A. Howes and Vivier (121), however, could not prevent the formation of gastric lesions in rats by adding cod liver oil to a vitamin deficient diet. Zucker and Berg (271) reported that hyperplastic gastric lesions were produced in rats by very low dietary levels of calcium. At moderately low (but not very low) calcium levels, administration of vitamin D decreased the incidence of these gastric lesions.

Harde and Kobozief (111, 112) found that the administration of calcium plus vitamins A and D lowered the incidence of spontaneous mammary tumors in a susceptible strain of mice. Barbiroli (20) found that the injection of a vitamin D concentrate had little influence on the growth of transplanted Ehrlich mouse carcinoma. Vollmar (251) found no difference in the growth of normal or cancer tissues in tissue culture upon the addition of vitamin D to the culture medium.

Huzita (122) found that a surplus of vitamin D did not affect the growth of a transplanted rat sarcoma, but slightly increased its sensitivity to Xrays. Sumi and Nakahara (232) showed that ether extracts of several tumor tissues were able to cure rickets in rats, indicating the presence of vitamin D in these tissues.

Blum and his associates and others have produced tumors in mice by extended exposure to low intensities of ultraviolet light. Blum and Lippincott (31) considered the possibility that the irradiation might have resulted in excessive production of vitamin D. They were, however, unable to detect any pathological symptoms of vitamin D hypervitaminosis.

5. Vitamin E

Vitamin E has been identified with no enzyme system or comparable cellular function. However, extensive changes in tissue morphology, particularly of muscle, occur in vitamin-E-deficient animals (Wolbach and Bessey, 261). There is an increased metabolism of tissues of vitamin-E-deficient animals, which is restored to the normal level soon after the administration of α -tocopherol. This effect on tissue metabolism suggests that α -tocopherol in some way functions as a regulator of metabolism. The relation of vitamin E to the function of certain endocrines has been demonstrated, and effects of its absence or surfeit might be sought on the basis of induced hormonal imbalances.

Most of the work on the relation between vitamin E and malignant growth has been carried out using wheat germ oil, a rich source of vitamin E. However, other factors are also present in these preparations, and much of the conflicting data regarding the effect of vitamin E on malignant growth may be attributable to such unknown factors.

Deficiencies of vitamin E have been shown to cause hypertrophic changes in tissues, and in fact to lead to the development of malignant tumors.

Thus Adamstone (7) has found lymphoblastomas in chicks fed a diet treated with ferric chloride to destroy vitamin E. Adamstone (6, 8) also found ulceration and tumor formation in the intestines of chicks fed similar diets. The type of lesions developed by chicks depended upon the source of supplementary vitamin D, different results being obtained in diets containing cod liver oil and haliver oil. Bishop and Morgan (29) found deciduomata in rats kept on a diet low in vitamins A and E, and Evans (80) showed that rats in pseudopregnancy on a low vitamin E diet, frequently developed deciduomata.

Rowntree and his associates (71, 198, 200, 201) claimed to have produced malignant transplantable sarcomas by feeding crude wheat germ oil to rats. This result, however, has not been obtained in numerous other laboratories (19, 32, 40, 43, 65, 66, 68, 70, 81, 94, 109, 110, 194, 204).

Davidson (62, 63, 64) found that the induction of tar carcinomas in mice was somewhat lower in mice on a diet supplemented with wheat germ oil. Cameron and Meltzer (52), however, observed only a delay in the appearance of tar-induced tumors in mice. Severi (210, 211) had equivocal results which suggested that mice on a high vitamin E diet were somewhat more susceptible to tar-induced tumors than mice on a low vitamin E diet. Haddow and Russel (109) found that addition of wheat germ oil to the diet had no influence on the induction of tumors in mice painted with 3,4-benzpyrene, and Severi (212) has had similar results. Carruthers (53) found that the vitamin E level of the diet had no influence on the production of tumors in mice by injection of methylcholanthrene. The incidence of spontaneous mammary tumors in strain A female mice was somewhat higher on a vitamin-E-deficient than on a maintenance E diet (56). Wheat germ oil had no influence on the development and growth of the Ehrlich mouse adenocarcinoma (Engel, 76) or on the Jensen rat sarcoma (Marchesi, 155; Zagami, 270).

Bryan and Mason (43) maintained Walker 256 mammary carcinomas in vitamin-E-deficient rats for 22 consecutive implantations, and found no alteration in the rate of growth, transplantability, or histologic appearance of the tumor. Sugiura (227) found that wheat germ oil did not protect against the formation of hepatomas in rats on an unpolished rice diet fed *p*-dimethylaminoazobenzene.

Vollmar (251) found that vitamin E stimulated the growth of transplanted normal tissue and inhibited the growth of tumor tissue in tissue culture. Von Euler and von Euler (78, 79) found that the vitamin E content of Jensen rat sarcoma and the Brown Pearce rabbit carcinoma was relatively high as compared to most normal tissues.

The foregoing brief resume of the literature indicates that there is no general consistency in the effect of vitamin E on malignant growth, and at

the present time few valid conclusions can be drawn regarding this relationship. Adamstone (9), however, has collected and considered evidence showing that vitamin E may be involved in the metabolism of substances containing the phenanthrene nucleus, *i.e.*, cholesterol, androgen, estrogen, progesterone, and many carcinogenic hydrocarbons. The evidence for this view is indirect but extensive. Various hormonal imbalances and malfunctions, particularly of the pituitary-gonad hormones, might possibly result from a deficiency or excess of vitamin E, and these could conceivably influence the formation of tumors in a fashion as yet unpredictable. The experiments of Lipschutz have shown that hormonal imbalances and malfunctions may bring about the formation of certain types of cancer.

6. Vitamin K

Vitamin K has been shown to function in the formation of prothrombin in the liver, but information as to how it influences this synthesis is still lacking. Vitamin K is a reversible oxidation-reduction system, and the suggestion has been made that it inhibits the lytic action of the liver cathepsins on prothrombin. Vitamin K is synthesized by bacteria, and failure to develop vitamin K deficiencies in rats is credited to its synthesis by the flora of the intestinal tract. The presence of vitamin K in microorganisms makes it very likely that its activity is not limited to its influence on the synthesis of prothrombin, but that it is also active as the prosthetic group or in some other capacity associated with enzymatic action.

Few experiments have been performed on the influence of vitamin K on the incidence and growth of experimental cancer. Fieser (86) suggested that the detoxification of natural and unnatural carcinogens might be brought about by their combination with suitable disulfide containing proteins. Prothrombin appears to be active in blood clotting only in the S-S form, and Fieser suggested that it might function as a detoxifying agent for protection of the body against incidental carcinogens. This would suggest that vitamin K would have a protective action against carcinogenesis by maintaining the blood prothrombin at a high level. It is interesting to recall that patients with gastric and other cancers have a lowered level of serum prothrombin (Abels, *et al.*, 4).

III. ABNORMAL METABOLISM OF VITAMINS IN CANCER

The presence of tumors in experimental animals has been shown to influence the activity and function of enzyme systems in organs remote from the site of the tumor. Greenstein and Andervont (105a) found that the catalase activity of liver in rats bearing transplanted tumors was very much less than that of the normal rat liver. Robertson and Kahler (196) found

that the liver riboflavin was decreased in rats bearing transplanted hepatomas. Such systemic effects, if they also occur in humans, may have an influence on the prognosis and treatment of cancer patients. The hormonal treatment of prostatic cancers developed by several investigators (see accompanying chapter by Dodds) is an example of the use of such indirect therapy in cancer.

Certain evidence has recently indicated that the metabolism of certain vitamins in patients with cancer may be somewhat deranged. Thus Abels and co-workers (3) reported that the plasma carotin and vitamin A was significantly lower than normal in patients with gastro-intestinal cancer and other forms of cancer. The explanation for the low plasma vitamin A was thought to be due to the inability of the liver to store vitamin A or to form it from carotene. This suggestion was supported in a further study (4) in which it was shown that patients with gastro-intestinal cancer had a high incidence of hepatic dysfunction as determined by a number of criteria. It is significant that the changes in plasma prothrombin paralleled the change in plasma vitamin A. This may possibly indicate an abnormal metabolism of vitamin K in cancer patients. Grant (104) also found that cancer patients tended to have low blood vitamin A levels, and that as the cancer progressed the blood vitamin A fell to even lower concentrations. This effect of cancer on plasma vitamin A is an exceedingly interesting development, and a thorough investigation of the generality of the phenomenon is desirable.

There is some evidence also that the metabolism of vitamin C is altered in patients with cancer. Antes and Molo (17), Gaechtgens (93), Spellberg and Keeton (223), and Minor and Ramirez (162) have reported that patients with advanced cancer had a greater "vitamin C deficit" than normal. In cancer patients given an excess of ascorbic acid daily (*ca.* 500 mg.), there is a longer time interval than normal before the urinary excretion of ascorbic acid rises to a saturation plateau. Patients previously on a scorbutic diet take still longer to develop tissue saturation. After saturation of the tissues by continued administration of excess ascorbic acid, it was found (93, 162, 223) that the daily utilization (the difference between ascorbic acid administered and that recovered in the urine) was somewhat higher in cancer patients than in normal controls. However, it should be pointed out that a decreased excretion of ascorbic acid is also encountered in other diseases. Stepp and Schroeder (224) had similar results with normal and tumor-bearing mice. It was suggested that the difference in daily utilization was due to the excessive utilization of ascorbic acid by the cancerous tissue. Should this be the case, the tissues of tumor bearing animals should contain less ascorbic acid than non-tumor bearing controls. A slightly lowered ascorbic acid content has been found in the tissues of rats

and mice with cancer (Sure, *et al.*, 234). Watson and Mitolo (254) and Woodward (265), however, found no such decrease in the tissues of tumor-bearing rats.

The metabolism of thiamine in the white cells of leukemia patients appears to be different from normal white cells. Abels, *et al.* (1) found that the thiamine content of the leukocytes and platelets of patients with leukemia was several times higher than the thiamine in normal leukocytes and platelets. The concentration of a pyrimidine which accelerates yeast fermentation, and which is probably a metabolite of thiamine, formed an abnormally small percentage of the total thiamine content of leukemic white cells. It was suggested that the difference in the concentration of thiamine and its metabolite in normal and leukemic white cells was due to impaired utilization of thiamine by the cells, and not to increased ingestion, faulty excretion or relative age of the cells involved.

Other differences between normal and leukemic leucocytes have been observed. Thus ten times as much vitamin C was present in human leukemic white cells as in normal white cells (Butler and Cushman, 51).

The old concept that cancerous tissue might have an abnormal or excessive vitamin requirement or metabolism finds a certain amount of support in the observations on vitamin A, ascorbic acid, and thiamine just reviewed. However, the evidence is still fragmentary, and any hope of controlling cancer based on a supposed excessive vitamin requirement of cancer tissue (Laurence, 145) must await the demonstration of such excessive requirements.

IV. THE RELATION OF VITAMINS TO LESIONS AND CANCER OF THE INTESTINAL TRACT

Certain evidence has accumulated to show that vitamin deficiencies may play a rôle in the development of intra-oral and gastro-intestinal cancer, an observation of special interest since approximately half of the cancer deaths in 1940 resulted from cancer in the buccal cavity and the gastro-intestinal tract. In recent articles Martin and Koop (157) and Abels, *et al.* (5) have shown that B complex deficiencies are probably closely associated with intra-oral cancers. They found that many of the oral cancer patients coming under their observation had degenerative oral mucus membrane changes which were in large part alleviated by the administration of B complex vitamins. Martin and Koop believe that supplementary vitamin therapy is a most important factor in the treatment and prevention of oral cancers. Clinical evidence also indicated that B complex deficiencies might cause degenerative changes in the whole of the gastro-intestinal tract.

Fibiger (82, 83, 84, 85) was one of the first to produce experimental gastric lesions. This investigator succeeded in producing benign hyperplasia

and also what he believed to be squamous cell carcinomas in the forestomach of rats by feeding them the somewhat unusual diet of white bread and water supplemented with cockroaches infected with a parasitic nematode, *Gongylonema neoplasticum* (*Spiroptera neoplastica*). He believed that the parasites were responsible for the lesions, but Passey, Leese, and Knox (186), repeating Fibiger's experiments, obtained similar but milder lesions in rats not infected with the nematode. They pointed out that the diet was deficient in many vitamins and performed experiments showing that a lack of vitamin A was an important factor in the production of the lesions. Pappenheimer and Larimore (184, 185) observed similar lesions in rats fed a rachitic diet deficient in not only vitamin A but also other factors. Wolbach and Howe (262) found stratified keratinizing epithelium in the alimentary tract of rats on a low vitamin A diet. Fujimaka (91) claimed the appearance of gastric carcinoma in rats on an A-deficient diet, but some doubt as to the diagnosis was brought out in an editorial accompanying the report. Tilden and Miller (243) found metaplasia in various epithelial tissues of monkeys and rats on an A-deficient diet. Fredericia, *et al.* (90) found proliferative changes in rats fed inadequate vitamin A. Cramer (59) in two experiments, done ten years apart, on the effect of vitamin A deficiency in rats observed severe gastric papillomas in the first and only mild lesions in the second experiment. No explanation for the difference in the results was apparent. Andervont (11) found that diets rich in vitamin A did not influence the formation of spontaneous adenomatous lesions occurring in the pyloric stomach of strain I mice, and concluded that this lesion was not due to a vitamin A deficiency. Beck and Peacock (24) observed gastropapillomas in rats fed heated fat. These animals developed vitamin A deficiency symptoms, and vitamin A administration was found to prevent the formation of the gastric lesions.

Lesions are also produced in rats deficient in other vitamins. Sharpless (214 to 219) has observed epithelial hyperplasia in rats on a deficient diet, and that riboflavin, pyridoxin, choline, and cystine are important factors preventing this hyperplasia. Dalldorf and Kellogg (60) found that a large proportion of thiamine-deficient rats had gastric ulcers. McCarrison (149) observed a stomach cancer in a monkey on a B-complex-deficient diet and Findley (88) reported lesions in rats due to thiamine and riboflavin deficiencies. Howes and Vivier (121) found gastric lesions in rats fed the deficient diet of Pappenheimer and Larimore and showed that these lesions were prevented by addition of whole yeast to the diet. Brunswick and Rassmussen (42) fed rats a variety of unbalanced diets with and without supplementary vitamins A, C, D, thiamine, and riboflavin, and obtained ulcero-papillomas of the stomach. The lesions did not appear to be a direct result of vitamin inadequacies, but to be referable to a general mal-

nutrition. Sugiura (228) found that rats fed on a polished rice diet developed epithelial hyperplasia of the forestomach while those fed unpolished rice did not. The difference between these diets is largely a matter of their content of the vitamin B complex. Fasting, deprivation of protein, and dietary conditions other than vitamin deficiency *per se* have been shown to cause gastric lesions in the rat (Morris and Lippincott, 171). The recent reviews of Cramer (59), Sugiura (228), and Klein and Palmer (139) contain more information on the relation between diet and gastric lesions.

From the results just briefly summarized one certainty emerges—the nature of the diet and the vitamin content may influence the development of degenerative and hyperplastic lesions of the forestomach of the rat, mouse, and monkey. However, other experimental animals have been refractory to the production of gastric lesions by dietary means. It is noteworthy that most of the lesions developed in rats and mice have been in the squamous epithelium of the forestomach, rather than in the glandular epithelium of the pyloric stomach. This latter type of epithelium is analogous to that found in the stomach of man. It is also worthy of note that few if any of the lesions developing in rats as a result of dietary deficiencies have been malignant tumors, and, although it may be tempting to believe that these are precancerous changes, this is scarcely justified at the present time.

V. TUMORS INDUCED BY *p*-DIMETHYLAMINOAZOBENZENE

1. *Effect of Dietary Vitamins on Tumor Production*

A great deal of progress, even if not final definition, has been made in studies of the effect of dietary vitamins upon not only the development of hepatic tumors in rats fed certain azo dyes, but also related enzyme and vitamin analyses of the tumors, and of their metabolism. Probably more biochemical work has been done on the azo-dye-induced hepatic tumors than any other type of tumor; at the same time, it is to be kept in mind that for the present it is a special problem confined largely, if not entirely, to hepatic tumors; perhaps only liver can effectively act upon, and be acted upon by the carcinogenic azo dyes. The demonstration of the carcinogenic action of the azo dyes arose from attempts to use scarlet red to stimulate epithelial proliferation. Japanese workers found that *o*-aminoazotoluene, the active constituent of scarlet red, would produce liver tumors when administered orally to rats fed polished or brown rice a diet deficient in protein, salts, and B complex vitamins (Yoshida, 268, 269; Sasaki and Yoshida, 205; Kinosita, 137). Another azo dye, *p*-dimethylaminoazobenzene or "butter yellow," later proved to be even more carcinogenic for rats (137), and most of the subsequent work has been carried out using this compound.

It was early recognized by the Japanese workers that the nature of the diet was of primary importance in determining whether or not animals fed *p*-dimethylaminoazobenzene would develop liver cancers. Kinoshita (138), Ando (13), and Nakahara, Fujiwara, and Mori (174) found that the addition of about 10 per cent dried yeast to the basal *p*-dimethylaminoazobenzene-rice diet markedly diminished the incidence of liver tumors in rats. Nakahara, Mori, and Fujiwara (176, 177) then showed that dried liver protected rats on a polished rice-*p*-dimethylaminoazobenzene diet practically completely against liver tumors. The carcinogenic action of orally administered *p*-dimethylaminoazobenzene and *o*-aminoazotoluene, and the protection by yeast, liver, and other dietary agents have now been observed by a great many investigators (14, 15, 16, 18, 108, 117, 134, 135, 154, 160, 163, 164, 165, 166, 167, 180, 206, 221, 227, 230, 231, 244, 245, 257, 258, etc.).

The efficacy of substances like liver and yeast in protecting against *p*-dimethylaminoazobenzene tumors led to the hypothesis that certain of the B complex vitamins might be the effective agents. Nakahara, Mori, and Fujiwara (175, 177) found that the addition of known vitamins of the B complex alone, or in various combinations, showed but slight tendency to prevent the formation of *p*-dimethylaminoazobenzene tumors in rats on a polished rice diet. However, it may now be said that the negative results of these workers were due in large measure to inadequate vitamin dosages and to other non-protective features of the diet (low protein, possibly low salts).

Kensler, *et al.* (135) demonstrated a pronounced protection against the formation of these tumors when large doses of riboflavin, especially in combination with casein, were added to the diet. This result has also been obtained by György, Poling, and Goldblatt (108), Antopol and Unna (18), Burk, *et al.* (46), Miller, *et al.* (160) Miner, *et al.* (161), and Reimann, Stimson, and Medes (192a). The anticarcinogenic action of riboflavin against liver tumors induced by *p*-dimethylaminoazobenzene feeding becomes increasingly apparent over the range 10 to 50 (to 200) γ per rat per day, which is somewhat greater than that needed for the normal growth and maintenance of the rat (ca. 20 γ). The anticarcinogenic action of riboflavin depends to a great degree upon the concentration of casein (or other protein in the diet) being more effective at high casein levels. Riboflavin has had only an anticarcinogenic action, if any, in primary azo dye hepatoma formation thus far investigated, although as already indicated, a promoting action on the transplanted hepatoma 31.

In an investigation to ascertain whether biotin also was protective against the production of *p*-dimethylaminoazobenzene hepatomas, du Vigneaud, *et al.* (245) and Burk, *et al.* (47) reported that pure biotin actually

promoted the formation of these tumors in rats fed a highly protective riboflavin-casein diet. In support of this reported procarcinogenic action of biotin on these induced liver tumors are observations that dietary egg white decreases liver damage and acts anticarcinogenically in rats fed *p*-dimethylaminoazobenzene (245, 47, 160, 221). Egg white contains a protein, avidin, which combines with dietary biotin, as well as biotin synthesized in the intestinal tract, and renders it unavailable to the animal. Therefore the protective action of egg white in reducing the formation of liver tumors may well be referable to the lowered biotin availability which its content of avidin entails, although riboflavin, sulfur, and nitrogen in the egg white must also be kept in mind here. The procarcinogenic action of biotin appears to develop over the range of about 0.1 to 1 γ or more per rat per day, and the counteracting anticarcinogenic action of egg white occurs at dietary levels of 0.3 to 3 g. dried egg white (2-10 units of avidin) or more per rat per day. The promoting action of excess biotin (and also riboflavin) on reversal of regression of transplanted hepatomas (242) has been noted earlier. It is apparent that pure biotin has thus far always had a procarcinogenic, and egg white or avidin an anticarcinogenic, action. Certain crude biotin preparations, however, have been found to have no effect or to have had a protective action against *p*-dimethylaminoazobenzene tumor formation (245). This was very likely due to other overbalancing protective factors in the crude preparations.

Although the foregoing studies on biotin are highly significant, much more information is necessary in order to determine whether the procarcinogenic action of biotin is a general phenomenon or is restricted to *p*-dimethylaminoazobenzene tumors under a particular set of experimental conditions, possibly involving intestinal tract microflora.

In addition to riboflavin and biotin, certain other B vitamins have been observed to influence the formation of hepatomas in *p*-dimethylaminoazobenzene-fed rats. Pyridoxin has been found to have a procarcinogenic action, analogous to that exerted by biotin (Miner, *et al.*, 161) and inositol has been reported to exert a similar effect (Miller, *et al.*, 160; Burk, *et al.*, 46). In a highly protective diet, a procarcinogenic effect of *p*-aminobenzoic acid has been observed (Burk, *et al.*, 46), while in a non-protective diet *p*-aminobenzoic acid was anticarcinogenic (47). Although nicotinic acid had little effect on the production of hepatomas in rats on a brown rice-*p*-dimethylaminoazobenzene diet, it appeared to slightly increase the protection in the same diet supplemented with riboflavin (Kensler, *et al.*, 135).

Several workers have investigated the effect of choline on the production of hepatomas in rats fed *p*-dimethylaminoazobenzene. The addition of choline or lecithin to the usual (non-protective) brown rice-*p*-dimethylaminoazobenzene diet of the Japanese had no effect on the incidence of

p-dimethylaminoazobenzene tumors in rats (Burk, *et al.*, 46). However, the addition of choline to a fairly protective riboflavin-casein-cystine-supplemented diet afforded additional protection (Burk, *et al.*, 46). White and Edwards (257, 258) found that cystine added to a low protein-high fat-*p*-dimethylaminoazobenzene diet markedly increased the production of hepatomas. The addition of choline to this diet, while it decreased the extensive cirrhosis, had no appreciable effect on the final incidence of tumors (258). Although no data on the induction (latent) period were given in the latter paper, it is quite probable that the induction period was increased by the choline, in harmony with findings of György, Poling, and Goldblatt (108), who obtained a (delaying) protective action by combined choline and cystine in a rice diet. Miller, *et al.* (160) and Dyer (72) observed a definite procarcinogenic action of choline. Thus choline has been observed to act both procarcinogenically and anticarcinogenically. Cystine, too, has been shown to have both a favorable and an unfavorable influence on hepatoma formation. A protective action has been observed by Burk, *et al.* (46) and by György, Polling and Goldblatt (108), while under other circumstances a tumor promoting action (decreased latent period) was found by White and Edwards (257, 258) and György, Polling and Goldblatt (108). In other experiments no significant influence of cystine on hepatoma production has been noted, (Miller, *et al.*, 160; Burk, *et al.*, 46; Mori, 165). The interesting interrelationship between choline, cystine, methionine, and methyl groups observed in general nutrition may be involved in the effects of these agents on hepatoma production. In general it appears that cystine and choline each act procarcinogenically when the diet is otherwise poorly protective (*i.e.* low riboflavin or protein) and anticarcinogenically when the diet is otherwise fairly protective, and especially when both are present together.

Positive indication that still other B vitamins influence the production or prevention of hepatomas induced by feeding *p*-dimethylaminoazobenzene has not been obtained although the possibility is by no means excluded. Of the other vitamins only vitamin E, A, and D have been tested. Sugiura (227) found that including wheat germ oil in an unpolished rice-butter yellow diet had no influence on the incidence of hepatomas. Cod liver oil, a rich source of vitamins A and D, had no protective action against *o*-aminoazotoluene induced hepatomas in rats in the experiments of Ando (16).

2. Changes in the Vitamin and Enzyme Content of Liver during Hepatoma Formation

It is now well established that riboflavin, niacin, and thiamine form the active groups of certain enzymes. If these vitamins influence the

production of hepatomas in rats fed *p*-dimethylaminoazobenzene, the vitamin content and activity of enzymes containing these vitamins might be expected to be influenced by *p*-dimethylaminoazobenzene feeding. Rather extensive studies have been made of the vitamin content and enzyme activity in normal rat liver, *p*-dimethylaminoazobenzene fed rat liver, induced and transplanted hepatomas. These studies constitute the most extensive investigation of biochemical changes in tissue accompanying its transformation into malignant growth yet at hand, and the results of many investigators are summarized in Table I.

The data of Kensler, Sugiura, and Rhoads (134) and of others (128, 159, 187, 188, 196) given in Table I show that the riboflavin content of the liver of azo-dye-fed rats is lower than the normal level, and that the induced or transplanted liver tumors contain even less riboflavin. Yeast feeding prevents the decrease in liver riboflavin (134), and also, as has already been brought out, protects against the development of cancer. The feeding of *p*-dimethylaminoazobenzene to rats causes a primary increase in urinary riboflavin excretion followed by a progressive decrease until markedly sub-normal values are found (134). These observations indicate that the susceptibility to and development of *p*-dimethylaminoazobenzene hepatomas is paralleled by the development of a liver riboflavin deficiency. The activity of the flavin containing enzymes, so far as they have been studied, also drops in *p*-dimethylaminoazobenzene-fed rat livers and tumors (Table I).

The biotin, pantothenic acid and pyridoxin content of liver does not appear to be lowered by *p*-dimethylaminoazobenzene feeding (188, 189, 238), although the concentration in the induced or transplanted tumors themselves is much lower than in the normal liver (188, 189, 238, 255, 256). The concentration of niacin falls slightly in *p*-dimethylaminoazobenzene-fed rat livers and somewhat more in the induced tumors (188, 237). The total drop is, however, much less than the decrease in the niacin containing carrier, coenzyme 1 (134). Thiamine content is about the same in liver and hepatoma (159, 188). Studies on the cocarboxylase content of these tissues would be highly desirable. The folic acid and inositol contents of normal and *p*-dimethylaminoazobenzene-fed rat livers and hepatomas were not found to differ significantly (188).

The vitamin A content of liver shows a pronounced drop in rats fed *p*-dimethylaminoazobenzene, and vitamin A is completely lacking in tumors induced by this agent (Goerner and Goerner, 99). Other carcinogens also cause a decrease in hepatic vitamin A.

Studies on the vitamin C content of livers and primary and transplanted tumors arising in azo-dye-fed rats have been made by Masayama, *et al.* (158), by Iki (124), by Fujiwara, Nakahara, and Kishi (92), and by Robert-

TABLE I

Vitamins, Enzymes, and Metabolism in Livers and Hepatomas of Rats Fed p-Dimethylaminoazobenzene or o-Aminoazotoluene

Liver Constituent	Reference No.	Units*	Normal liver	Liver from azo dye fed rats	Liver adjacent to induced hepatomas	Primary induced hepatomas	Transplanted hepatomas
Riboflavin	134	γ /g. DW	170	70	—	33	—
do.	159	γ /g. DW†	44	—	27	14.5	—
do.	187	γ /g. DW	90	—	72	28	—
do.	196	γ /g. DW	98	—	—	20	27
Biotin	189	γ /g. DW	3.23	—	2.4	0.7	—
do.	255	do.	3.45	—	3.5	1.4	—
do.	256	do.	4.48	—	4.5	2.0	—
Thiamine	159	γ /g. DW†	6.7	—	13	10.5	—
do.	188	γ /g. DW	25	—	17	20	—
Niacin	237	γ /g. DW	580	—	420	230	—
Pantothenic acid	238	γ /g. DW	273	—	240	74	—
Pyridoxin	188	γ /g. DW	6.1	—	7.0	2.5	—
Inositol	188	γ /g. DW	1770	—	1530	2380	—
Folic acid	188	γ /g. Δ	20	—	25	22	—
Vitamin A	99	USP units/g. DW	400	—	100	0	—
Ascorbic acid	91	mg./g. DW	0.18	—	—	—	0.40
do.	124	do.	0.26	0.30	0.17	0.15	—
do.	158	do.	0.21	0.30	0.20	0.13	—
Coenzyme 1	134	γ /g. DW	1390	500	—	150	—
Flavine adenine dinucleotide	213	γ /g. DW	61	—	—	—	61
L-amino acid oxidase	213	% normal	100	—	—	—	63
Xanthine dehydrogenase	106	% normal	100	—	—	—	50
Oxygen consumption	45	mm. O ₂ /hr./mg. DW	6.0	6.5	7.7	6.4	—
do.	134	do.	8.8	9.5	—	9.5	—
do.	178	do.	11.5	11.5	—	11.8	—
Anaerobic glycolysis	45	mm. ³ A/hr./mg. DW	1.0	3.1	2.3	12.1	—
	178	do.	3.2	10.5	—	18.8	—
	181	do.	7.1	10.8	—	16.7	—

* All values expressed in terms of dry weight (DW).

† Calculated from original wet weight values by assuming 30% and 18% dry weight for liver and tumor respectively.

Δ Micrograms of material with "potency" 40,000.

son (195). In general the precancerous changes of the liver were accompanied by a somewhat higher concentration of ascorbic acid than normal liver. Induced liver tumors seem to have a somewhat lower,

and transplanted liver tumors a somewhat higher, ascorbic acid content than normal liver.

The significance of the changes in the vitamin contents of the hepatomas as compared to the normal liver from which they are derived is as yet rather difficult to determine. Such differences may be due entirely to the change or loss in function involved, and not causally connected with the development of the tumor. It is well established that the liver is the great storage organ for certain, and probably most, of the vitamins, and, with the loss of this function accompanying the transformation of the liver into hepatoma, it might well be expected that the vitamin content would drop. An illustration of this is the observation that, whereas the biotin content of hepatoma is much less than that of the liver, it is still much higher than most other tissues of the body.

A more delicate determination of the essential metabolic changes accompanying tumor formation and the influence of vitamins thereon would be obtained through the measurement of the activity of those enzymes which have vitamins as prosthetic groups. Where this has been done (coenzyme 1, cocarboxylase, *d*-amino acid oxidase, xanthine dehydrogenase, flavin adenine dinucleotide) there has been, in general, a decrease in activity which is less than the decrease in the corresponding vitamin content (cf. Table I). The concentrations of many cellular constituents in liver and in hepatomas, and the activities of several enzymes which are not known to have vitamin prosthetic groups, have been listed by Greenstein (105). Here, too, enzyme activity was in general found to be less in the tumors than in normal liver.

3. Changes in Metabolism with Tumor Formation

It is interesting to correlate the changes in enzyme and vitamin content with the changes in metabolism during the transformation of normal liver to hepatoma. It has been indicated that the activity of many of the enzymes involved in intermediary metabolism is reduced in the formation of liver tumors due to feeding of *p*-dimethylaminoazobenzene. How, then, is the over-all metabolism affected? Surprisingly enough, the respiration rate of the tumors is about the same as that of normal liver, whereas the anaerobic glycolysis is many times higher than in liver (cf. Burk, *et al.*, 44, 45; Nakatani, Nakano, and Ohara, 178; Orr and Stickland, 181; Kensler, Sugiura, and Rhoads, 134) (see Table I). This undoubtedly indicates that there is normally a considerable excess in the potential activity of the liver metabolism enzymes, and that there can be a large reduction in their concentration without lowering them to a limiting level. An example of this phenomenon is given by the observations that normal tissues have a large "excess oxidation capacity" whereas cancer tissues do not

(Craig, Basset, and Salter, 58). We have made studies based on these observations which show that the respiration of normal liver is stimulated 200 to 400% by the addition of *p*-phenylenediamine or sodium succinate. This large stimulation indicates that the liver cytochrome-cytochrome oxidase system is capable of acting much faster than the slower rate-limiting reaction in the respiration chain can transport the hydrogen or electrons to this system. All cancer tissues that we have examined have showed only slight stimulation of respiration upon the addition of *p*-phenylenediamine and practically none with succinic acid. This is in agreement with observations that hepatomas contain less cytochrome C, cytochrome oxidase, and succinic oxidase than normal liver. This reserve activity of the normal liver enzymes makes it possible for them to be substantially diminished without influencing the rate of over-all metabolism, and possibly is the explanation for the fact that hepatomas may show a lowered activity for most enzymes and yet a higher anaerobic metabolism than normal liver.

4. *The Metabolism of p-Dimethylaminoazobenzene in the Rat*

There is a strong possibility that the carcinogenic action of *p*-dimethylaminoazobenzene is due, at least in part, to interference with enzymes. *p*-Dimethylaminoazobenzene itself does not appear to be very toxic, and it seems likely that it may be metabolized in the liver to form more toxic derivatives.

Stevenson, Dobriner, and Rhoads (226) have studied the fate of orally administered *p*-dimethylaminoazobenzene and have demonstrated that the azo linkage is broken, liberating, presumably, aniline and dimethyl-*p*-phenylenediamine. These two compounds were not isolated, but the closely related *p*-aminophenol and *p*-phenylenediamine and their acetyl derivatives were isolated from the urine of rats fed *p*-dimethylaminoazobenzene. Jacobi and Baumann (126) found that feeding of the latter prevented the development of kidney lesions in young rats on low choline diets. This indicated that the demethylation of *p*-dimethylaminoazobenzene allows it to serve as a source of labile methyl groups. This factor might be involved in the pro- and anti-carcinogenic action of choline already discussed.

The full significance of the split products of *p*-dimethylaminoazobenzene metabolism is not yet known. It is not yet possible to determine with certainty whether the active carcinogenic agent is the original dye or one of its derivatives, since the dye itself is relatively non-toxic to enzymes (131). The oral administration of the most interesting derivatives has not yet been successfully carried out to see if they are carcinogenic. Since they are water soluble whereas the parent compound is not, the

failure to demonstrate a carcinogenic action of the derivatives will not constitute conclusive evidence that they do not function as the actual carcinogenic agents in the liver.

5. The Action of *p*-Dimethylaminoazobenzene and Its Derivatives on Enzymes Systems

The observation that the content of niacin containing coenzyme 1 in livers and tumors from azo-dye-fed rats was much reduced (134) led Kensler, Dexter, and Rhoads (132) to investigate the influence of *p*-dimethylaminoazobenzene and certain derivatives which result from its metabolism on the activity of this coenzyme *in vitro*. It was found that the split products of *p*-dimethylaminoazobenzene and other azo dyes were highly toxic to an enzyme system in which the concentration of coenzyme 1 was the limiting factor. The toxicity of the various split products was directly related to the carcinogenicity of the parent compounds, the stability of the intermediate free radical, and the ease of oxidation of the reduced compound. Potter (192) extended this study to the urease and succinic oxidase systems and found that the oxidized derivatives were highly toxic while the reduced diamines were not. He suggested that the mechanism of inhibition was a combination of the oxidized split product with the sulfhydryl groups of the enzymes. *p*-Phenylenediamine and dimethyl-*p*-phenylenediamine have also been shown to be toxic to a yeast carboxylase preparation, and the inhibition is overcome by adding an excess of thiamine-containing cocarboxylase (136). The *in vitro* respiration of slices of liver and other tissues has been shown to be inhibited by split products of dimethylaminazobenzene (133). The flavin enzyme xanthine oxidase was found to be inhibited by the *p*-phenylenediamine and dimethyl-*p*-phenylenediamine, while cytochrome oxidase, urease, alkaline and acid phosphatase, and *D*-amino acid oxidase were slightly or not at all affected. (131; 133) Rusch and Kline found that *p*-dimethylaminoazobenzene and other carcinogens inhibited the oxidation of phospholipoid emulsions brought about by ascorbic acid (202).

6. General Remarks on Diet and *p*-Dimethylaminoazobenzene Induced Hepatomas

The established fact that dietary vitamins can markedly influence the production of liver tumors induced in rats by azo dye feeding, provides a most interesting entrance point into a study of the relation of diet and vitamins to malignant growth. However, the interrelationships are still exceedingly complex, and considerable study will be necessary to determine the full mechanisms of action of various vitamins upon the carcinogenic action of *p*-dimethylaminoazobenzene. Probably each effective vitamin

will have a different function and an immediately different mechanism by which it brings about its effect.

The induction of a tumor in the liver represents the *net result* of the transport of the carcinogen to the liver, its effect on the metabolism of the cells, the rate of its detoxification by the cells, protective and immunological reactions, the growth of the tumor to a demonstrable size, etc. Since diet may influence any of these factors, it is clear that a protective agent may operate in any of a number of ways. An agent, vitamin or otherwise, involved directly or indirectly in the detoxification of azo dyes might be expected to protect against the formation of hepatomas induced by them. Agents which tend to cause or to prevent abnormal changes in liver histology or function might also be expected to have an effect on carcinogenesis due to azo dye feeding. However the action of an agent is not always predictable on these grounds. White and Edwards (257, 258); for example, found that the feeding of cystine, a material which unquestionably aids in detoxification of *p*-dimethylaminoazobenzene (46), did not prevent, but actually promoted the formation of hepatomas. Also, the addition of choline to this *p*-dimethylaminoazobenzene-cystine diet not only markedly diminished the degree of cirrhosis but prolonged the induction (latent) period of hepatoma formation, even though it failed to yield in their diet any eventual protection against tumors.

It is evident that an adequate understanding and interpretation of the literature on *p*-dimethylaminoazobenzene tumors requires a detailed knowledge of dietary levels of a great variety of vitaminic and avitaminic components. A number of important conflicting reports are possibly resolvable if knowledge already at hand is applied, particularly that relating to the quantitative levels of biotin and riboflavin involved. Examples that may be cited are: the higher incidence of tumors on brown rather than white rice diets, obtained at a moderate riboflavin level by György (privately communicated), as compared with a higher incidence on white rather than brown rice diets reported by Japanese workers, understandable in terms of higher (procarcinogenic) biotin in the brown rice in the first instance, and lower riboflavin in the white rice in the second instance; the breakdown of protection by yeast at a 50 per cent level as compared with 15 per cent, understandable in terms of the higher level of biotin then introduced; the shorter induction period of tumor incidence on the Japanese rice diets compared to semi-synthetic diets supplemented with small amounts of yeast, understandable in terms of lower riboflavin levels; the failure of Nakahara, Mori, and Fujiwara to obtain extensive protection in a rice diet supplemented with members of the vitamin B complex then known, the fat soluble vitamins, inorganic salts, and fish protein, supplied singly and in combination (but, as already indicated, some in inadequate

quantities). These cases are much better understood upon due consideration of the probable levels of biotin and riboflavin involved, as are many similar cases (in particular the complete protection afforded by 5 to 15 per cent dried liver) when the levels of sulfur, nitrogen (protein), and methyl groups as well as other vitamins are taken into consideration also. The (anticarcinogenic) riboflavin level probably influences the effective (procarcinogenic) biotin level, and *vice versa*. The antagonism between biotin and riboflavin is, unlike that between biotin and avidin, very probably indirect, almost certainly involving no direct chemical reaction. It probably operates through the opposed metabolic influences exerted by biotin and riboflavin, the former being fundamentally fermentative (in the direction of tumor metabolism) and the latter fundamentally oxidative (in the direction away from tumor metabolism).

Practically none of the foregoing dietary factors can be said as yet to have been applied to distinguishing between tumor initiation and tumor development in growing or adult animals (as distinguished from dietarily-stunted young animals), and this is an important problem of the future. Likewise, whether these dietary factors might be so varied as to produce hepatomas in the absence of azo dye, remains to be investigated, but their now evident multiplicity indicates that this possibility should be seriously considered, directly or indirectly in future experimentation. Application to human cancer of information obtained with the azo dye tumors is likewise a problem for the future, although certain aspects are definitely under attack at present.

VI. THE VITAMER CONCEPT AND FUTURE RESEARCH

A need has been arising for a collective term to include all compounds which have a given activity. Thus, if two or more compounds will prevent the development of a given deficiency syndrome, both are vitamins, and in one sense they are one vitamin. The term "vitamer" has been proposed to refer to two or more compounds which relieve the same vitamin deficiency (49). There are, thus, A vitamers, D vitamers, biotin vitamers, thiamine vitamers, K vitamers, etc. It is evident that only the activity and not the chemistry of a compound is specifically implicated by the term vitamer. Another possible solution to the need for the collective term would be to delegate the term vitamin to describe an activity rather than to designate a compound. In view of the chronology of the subject, this would probably be an inconvenient procedure. Following the introduction of the term vitamer, the term hormomer has been used by Oliver J. Kamm in a forthcoming article, "The Mechanism of Hormone Action," to cover multiple active endocrine principles which are closely related chemically and physiologically; and similarly, for plant hormones there would be the

term auximer. Many other "mer"-suffixed terms likewise suggest themselves. Vitameric and other "meric" activity relationships may well involve chemical properties other than structure, such as various kinds of potentials (oxidation-reduction, dielectric constant), relative reactivities, etc.

A development which may be of utmost importance to vitamin-cancer research is the observation that inactive compounds related to particular vitamins may create deficiencies by competitively substituting for the active vitamin. Several examples of this type have now been described—for pantothenic acid (142, 222), for niacin (151), for vitamin K (183), for *p*-aminobenzoic acid (264), and for thiamine (266). Vitamin-analogues which may competitively produce deficiencies or raise the requirement for a particular vitamin may be termed competitive vitamers, or antivitamers. It is clear that a given compound may replace a vitamin in the growth of one organism and thus be an active vitamer, whereas it may be inactive and compete with the vitamin in another organism and be a competitive vitamer there.

This concept has been dealt with here since its application may offer what may be a very plausible means for producing differential vitamin deficiencies in growing and non-growing tissue. It seems possible that deficiencies of particular active vitamers may be produced with relative ease by the use of the corresponding competitive inactive vitamers. Rapidly growing tissue such as tumor presumably must synthesize its vitamin containing enzymes from available constituents, and thus might have its new growth made up in part with inactive forms of the vitamers which were administered. This might result in the inability of the tumor to grow. The more stable normal tissues might have much less tendency to replace their active vitamers by the inactive forms and thus could be affected to a much lower extent than the proliferating tissue. An illustration of this situation has been given by our studies with biotin and miotin. The former, but not the latter, is active in preventing egg white injury in experimental animals while both are active in promoting yeast growth (49). In rats fed avidin (which prevents the absorption of biotin from the intestinal tract without influencing the absorption of miotin) we have found that rapidly proliferating tissues show a much greater absolute, and percentage content of the inactive miotin. This is taken as an indication that the proliferating tissues have selectively taken up the inactive biotin vitamer which was present in relative excess. It is felt that the use of competitive vitamers for experiments on malignant growth is worthy of immediate research.

The driving motive of cancer-vitamin research is, of course, the hope of obtaining information leading to the eventual control of at least certain types of cancer. Work in this direction has thus far been carried out

along two lines—the production of vitamin deficiencies, and the addition of excess vitamins. The possibility that the initiation and growth of cancers in young growing animals can be selectively and effectively stopped by the mere omission of an essential dietary constituent is not great. The vitamin deficiencies reported in this review have, in both young and adult animals, almost without exception interfered with the cells of the host and of the tumor to about the same degree. However, in adult animals it is still possible that a selective inhibition of tumor growth might be obtained without inordinate injury to the host.

The use of supplementary amounts of vitamins to control cancer has a little more theoretical and experimental evidence in its favor. The vitamins doubtlessly play a rôle in the various protective mechanisms of the body, *i.e.* detoxification of harmful substances, repair of tissue injury, etc. A marginal vitamin deficiency may weaken these protective mechanisms and allow the initiation of neoplastic growth, which, after being started, is then largely subject to the same factors influencing the growth and maintenance of the cells of the host. The general trend of results discussed in the previous pages seems to indicate that the addition of a vitamin to a diet containing a marginal or extensive deficiency of the vitamin often (but not always) tends to decrease the incidence or percentage takes of the tumor, but to increase the growth rate of the tumors which do develop. The omission of particular vitamins from the diet tends to slow the growth of the tumor (and of the host) and may increase or decrease the tumor incidence or percentage takes. This latter variability may represent an influence on the balance of factors important in protection against the initiation of the cancer on one hand, and the factors favor in its growth, once started, on the other.

It is also evident from the work reviewed in previous pages that a given vitamin may not always be procarcinogenic or anticarcinogenic even with respect to a particular type of cancer or a particular carcinogenic agent. This was especially evident in the discussion of the effects of certain vitamins on the incidence of hepatomas in rats fed *p*-dimethylaminoazobenzene. Here, the concept of a dietary imbalance may throw some light on the occurrence of pro- and anti-carcinogenic effects by the same agent. If vitamins facilitate or stimulate the protective mechanisms as well as the growth of the tumor, it may readily be understood why either one of these two processes may become predominant depending upon the type of tumor, concentration of the vitamin, and other conditions.

It is now abundantly clear that not only may certain dietary supplements be procarcinogenic and others anticarcinogenic, but also that a given dietary component may act procarcinogenically under one set of circumstances and anticarcinogenically under another. Such double action by a given dietary

component, of promotion or protection depending upon circumstance, has been termed *amphicarcinogenic* (48), and substances or mixtures capable of evoking it, *amphicarcinogens*. The existence of a number of demonstrable amphicarcinogens makes it desirable to weld the concept of amphicarcinogenesis into our experimental thinking concerning the rôle of dietary balance in carcinogenesis, and into our interpretation of the bewildering array of dietary results now at hand.

With respect to genesis of hepatomas induced by the carcinogen *p*-dimethylaminoazobenzene fed to rats, the available data suggest that the amphicarcinogens recognizable at present include the substances choline, cystine (methionine by prediction), and probably *p*-aminobenzoic acid, and also various crude mixtures (certain liver concentrates, excessive dietary level of yeast); that biotin, inositol, and *p*-aminobenzoic acid, all members of the vitamin B complex, are procarcinogenic; and that riboflavin, avidin (egg albumen), and casein are anticarcinogenic, as are also, of course, certain crude natural materials (liver, yeast) or extractives thereof. The greater anticarcinogenicity of egg albumen compared to casein when both are at a given limiting, moderate level of 12 per cent (160), may be due to the 50 per cent greater methionine-cystine content as well as to its avidin or even riboflavin contents. This obviously raises the question of the equivalence of egg white and corresponding amounts of avidin. In this connection, it is to be kept in mind that quantitatively the efficiency of biotin-avidin combination in the intestinal tract is far from 100 per cent, and effective combination and inactivation of biotin in the intestinal tract requires a very great deal more than the stoichiometric amount of avidin.

The appearance of a visible tumor involves two more or less distinct aspects—genesis and growth. All studies involving the observation of large tumors at the end of a particular regimen actually determine, in a sense, the product of these two factors. Thus cells may undergo a malignant change, but if they do not grow, no tumor is formed. Similarly a tumor might be able to grow, but does not simply because it never gets started. It is exceedingly difficult to distinguish between these two processes, if, indeed, they can be separated. Certainly more studies on precancerous changes and the effects of vitamins on these changes are highly desirable and might elucidate the relation between the genesis of a tumor and its growth.

Vitamins may influence malignant growth through their effects on detoxification or other protective mechanisms just discussed, in the production or prevention of predisposing tissue changes discussed in connection with the effect of vitamins on the development of gastric lesions, or in other, even more indirect, ways. The observations of Tannenbaum

(235, 236) and many others clearly indicate that the limitation of caloric intake may decrease the incidence of a variety of different types of tumors. Since many of the diets used in creating vitamin deficiencies are less palatable or lead to a loss in appetite, some of the differences in tumor incidence may be directly due to the difference in caloric intake. This variable can and should be controlled by restricting the diet of control and experimental animals to the same level.

Vitamins may also affect cancer through indirect effects on the endocrine glands. Thus, if the growth and maturation of mice is stopped by a marginal deficiency of a particular vitamin or other essential dietary component, the development of secondary sex changes will be inhibited and susceptible strains of mice would not be expected to develop spontaneous mammary carcinomas. Other indirect effects of vitamin deficiencies or excesses on malignant growth are very likely, and should be considered in dietary cancer work.

The synthesis of certain vitamins (biotin, folic acid, vitamin K, and others) by the intestinal microflora in the rat and human has been established by a number of workers. The nature and type of intestinal synthesis is influenced by relatively small changes in the diet, which may lead to a definite effect on the nutritional status of the host as well as on the initiation and growth of malignant tissues. It is desirable to inject rather than to feed certain vitamins in some experiments in order to control what may be an important variable. The feeding of bacteriostatic agents such as sulfaguanidine, sulfasuccidine, tyrothricin, penicillin or certain phages, etc., also provides a means of evaluating intestinal synthesis as a factor in nutrition and in cancer development, and it is to be hoped that these sorts of experiments will soon be performed.

Another point worthy of mention is the recent tendency to use homologous normal and tumor tissues as a basis for comparing the vitamin content of the two types of tissues. This tendency is commendable, but some reserve must be exercised. This is particularly true when comparing the vitamin content of storage organs like the liver to the induced or transplanted hepatomas derived from liver. A more delicate method of determining functional changes in vitamin content would be to determine the activity of certain vitamin containing enzyme systems.

It may also be desirable to point out that adequate histological and pathological control is necessary for correct interpretation of many vitamin studies on cancer. Most tumors are composed to a very appreciable extent of normal cells mixed with the cancerous cells. Thus any quantitative difference between the cancer and the normal cells may be diluted out to a large extent. In recent unpublished studies on biotin and metabolism in normal and tumor cells, we have found that the conclusions

reached as to biotin content and metabolic behavior depend to a great degree on the histologic examination of the tissues in question and an estimation of the per cent of normal and cancer cells.

There is little doubt that the general principles of nutrition are about equally applicable to the growth of normal and malignant tissues. Thus it may be expected that any and all advances made in the science of nutrition can be applied to dietary cancer research. The reverse is equally true.

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Hormones in Cancer

By E. C. DODDS

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INTRODUCTION

The following article does not attempt in any way to be exhaustive, and strictly avoids the more nebulous and debatable aspects of the subject. Where the topic has been well ventilated in the past, only the very briefest summary will now be given.

While it has been hoped for the last 20 to 30 years that the study of endocrinology would provide some essential clue to the problem of malignancy, it is only recently that any clear-cut results have been obtained. A vast amount of uncritical literature concerning the effect of ablation of endocrine organs on the growth of tumors, the transplant-ability of tumors, the percentage of takes, etc. has appeared, but the results can usually be explained by a combination of the errors and various unknowns. It is safe to say that no clear-cut relationship has been shown between any particular endocrine gland and the growth of tumors.

The same applies to the effect of injecting extracts of organs into tumor-bearing animals. In practically all cases where definite results have been obtained, they can be explained by the fact that the injections were so toxic that they lowered the animal's general state of health and this affected the rate of growth of the tumor.

It is proposed to consider the following four aspects:

- I. Sex hormones as carcinogens.
- II. Estrogens and malignant disease of the prostate.
- III. Gonadotrophic substances and tumors of the testes.
- IV. Hormones and mammary tumors.

I.-SEX HORMONES AS CARCINOGENS

In some ways it is perhaps unfortunate that the structure of the sterols, the importance of the condensed carbon ring system compounds as carcinogens, and the elucidation of the structure of the sex hormones, all occurred

roughly in the same decade. The temptation towards theoretical speculation has proved too great for many biochemists and experimental cancer research workers, and a spate of papers have appeared which thoroughly disturbed the minds of clinicians who proposed to use the sex hormones. The tempting argument was that since the sex hormones belonged to the group of condensed carbon ring compounds, and so to the carcinogens, therefore the sex hormones—more particularly the unsaturated compounds such as estrone—were in themselves carcinogenic. A vast literature exists on this subject and it has been reviewed many times. The outcome, however, is now quite clear. In the first instance, the term 'carcinogen' should be restricted to those compounds which produce cancer when painted on the skin of mice over a prolonged period. It was by this test that Kennaway and his colleagues (23) made their classical discoveries in this field. Estrone when painted in a similar manner does not produce a local epithelioma and so cannot be described as carcinogenic in the same sense as are benzopyrene and similar compounds (Dodds, 2).

The properties of estrone and other estrogens which cause the development of carcinoma of the breast will be dealt with in Section IV.

II. ESTROGENS AND MALIGNANT DISEASE OF THE PROSTATE

It is in this field that the most striking developments have been made during the last four years. The following is a brief review of the situation up to date. For a detailed review of the literature up to January 1943 there is an excellent review by Haddow given in a discussion at the Faculty of Radiologists (11).

It will be remembered that many years ago castration was advocated for the treatment not only of carcinoma of the prostate, but also of benign enlargement. There can be no doubt that improvement, and in some cases regression and apparent cure, have been recorded. Nevertheless, the psychological effect of so drastic a treatment has given rise to many objections and rendered the method unpopular. It is only comparatively recently that the subject has been reopened, principally by American workers who will be quoted later. The theoretical background for such treatment lay in the observation that removal of the testes resulted in atrophy of the sexual organs including the prostate. It was assumed that this atrophy was the result of loss of the male sex hormone—an assumption which has subsequently been justified by studies with the pure crystalline hormone Testosterone.

The American worker Huggins and his colleagues (14, 15, 16, 17) have recently reopened this whole subject. They have shown that either direct castration, or what one might term indirect castration by the administration of estrogens, has a very valuable effect on the development of carcinoma

of the prostate. The American investigators had the advantage of the recently acquired knowledge that carcinoma of the prostate could be diagnosed and its progress studied by investigating the "acid" serum phosphatase. This arose through the work of the Gutmans and their collaborators (5, 6, 7, 8, 9) and this formed one of the most interesting and original observations in chemical pathology of the present day.

Very briefly, these workers found that prostatic tissue contains relatively large quantities of "acid" phosphatase—that is to say, of the enzyme capable of liberating phosphoric acid from glycerophosphate at a pH of about 5. They were able to prove that the prostate was the origin of the "acid" phosphatase of seminal fluid and also of male urine. While there is no understanding of the fundamental rôle of this "acid" phosphatase, it immediately occurred to the original workers that a study of its distribution might be of great interest in the various pathological conditions of the prostate gland, including carcinoma. It was shown, for example, that the "acid" phosphatase content of the prostate is very low until the age of puberty. Therefore Huggins, Scott, and Hodges (19) rightly assumed that the presence of this substance constitutes a secondary male sex characteristic. In confirmation of this thesis is the observation that the injection of testosterone propionate into immature *Rhesus* monkeys will cause within a few weeks a very great increase in the "acid" phosphatase content. On the basis of this observation, Scott and Huggins (27) suggested that the estimation of this substance in the urine and in the blood stream should be used as a basis for studying the activity of the prostate gland. In 1936, Gutman, Sprowle, and Gutman (10) showed that not only the prostatic tumor in carcinoma of the prostate, but also the secondary deposits contained large quantities of the enzyme. Examination of a number of patients with carcinoma of the prostate showed a very great increase in the enzyme in both the blood and the urine. This observation has been amply confirmed. It can be stated that a study of the quantitative distribution of this enzyme can be used as a method for the diagnosis and study of the progress of carcinoma of the prostate. It is clear, therefore, that anyone commencing observations on the treatment of carcinoma of this particular organ was in a much more favorable position than previous workers since they had at their disposal this excellent and accurate method for studying the effects of treatment.

It was quickly found that castration lowered the "acid" phosphatase content of both blood and urine, and that the injection of androgens caused a sudden increase in the concentration of the enzyme. This observation is known as the provocative test of Sullivan. 25 mg. of testosterone propionate are administered for 5 days. If the test is positive, there will be a distinct rise in the "acid" phosphatase content of the serum.

This does not occur in cases of benign prostatic hypertrophy, nor in other malignant conditions of the urinary tract. It is obvious that here is a very valuable method of distinguishing between malignant and benign enlargement of the gland.

It will be remembered that an enlargement of the prostate can be obtained in animals with estrone and that the enlargement can be reduced with testosterone. It would therefore appear that prostatic tissue is highly sensitive to sex hormones and particularly to estrone (Zuckerman, 28). Again it must be remembered that estrogens when given in sufficient doses will cause a practically complete inhibition of the anterior lobe of the pituitary, thus producing a castration effect. In the adult male rat it is possible with sufficiently large doses to cause gross atrophy of the testes in a short while. The indications, therefore, for experimenting with estrogens in the treatment of carcinoma of the prostate seemed very strong, since on the one hand the castration effect can be produced without the devastating psychological effect of removal of the testes, and on the other, the possibility of some sort of effect on the growth of the tumor directly can reasonably be expected. In 1941, Huggins and Hodges (18) showed that by the administration of estrogens the "acid" serum phosphatase could be reduced. They also showed that great improvement took place in the clinical condition of the patient. There now exists an extensive series of publications showing that following the administration of estrogens, either in the form of naturally occurring hormones given subcutaneously, or by the administration of the synthetic analogues stilbestrol and hexestrol given by mouth, virtual disappearance of the symptoms, not only of the enlargement of the prostate, but also of the secondary deposits, may occur. Thus has developed one of the most striking therapeutic researches of modern times. A series of references to recent papers on the subject may be found under the names of Herrold (13), Kahle and Maltry (20), Kearns (21, 22), Marquardt and Flaherty (26).

At present it is impossible to give a satisfactory explanation for these observations. The question naturally arises as to whether the action is exerted directly on the malignant tissue, or whether it has a castration effect due to inhibition of the anterior lobe. The subject certainly opens up a whole new field of research and forms one of the few clear-cut observations on the inter-relationship between the endocrine system and malignant disease.

The success of these experiments has led to a wholesale empirical experimentation with the synthetic analogues stilbestrol and hexestrol. These substances possess three characteristics which render them particularly attractive to the experimentally minded medical practitioner. First,

they are, by and large, non-toxic. Second, they are highly active by mouth, and third, they are cheap. A number of publications has appeared describing miraculous cures of carcinoma of the breast following the administration of either stilbestrol or hexestrol in large quantities. In the writer's opinion, the whole of this subject is in too nebulous a state to warrant detailed review at present. Unfortunately, practically all the recorded cases lack essential diagnostic factors such as histological sections, etc., and while it may be possible to state that the administration of estrogens has caused some changes in the rate of growth of certain carcinomata, it would be most unwise at the present juncture to recommend wholesale use of estrogens in this particular treatment.

III. GONADOTROPHIC SUBSTANCES AND TUMORS OF THE TESTES

While this is an old subject and little new material has come to light, purely theoretical biochemists tend to forget the very valuable practical results that can be obtained from examining the urine in cases of testicular tumor. The original observation was made by Ferguson (3, 4) who showed that in testicular tumors of certain types excessive quantities of gonadotrophic hormones were present. This was extended by a number of investigators, and one of the most careful researches on the subject was reported by Hamburger (12). The results are well summarized in the following table.

Hormones in Testicular Tumors.

No. of Cases	Diagnosis	Negative	Traces	Hypophyseal hormone	Chorionic hormone	Both hormones
42	Seminoma	13	4	25	0	0
2	Pseudo-seminoma	0	0	0	2	0
21	Mixed Epithelioma	3	1	0	14	3
10	Polycystic Teratoid	4	2	4	0	0

N.B. Table from "Recent Advances in Medicine" 10th edit. by Beaumont, G. E., and Dodds, E. C., Churchill, London, p. 272.

This table shows quite clearly that an accurate diagnosis of patients' testicular tumors can be made from a suitable analysis of the urine. The great importance of this test lies in the fact that an early diagnosis has a distinct bearing on the treatment, since it is generally agreed that tumors of the seminoma type are radiosensitive and therefore of good prognosis. On the other hand, the mixed epitheliomata are radio-resistant and usually end fatally in a period of months. One of the most interesting observations, as yet unexplained, is that even after removal of the seminoma either by operation or by radiation, the hypophyseal hormone continues to be excreted in large quantities although the patient appears normal.

IV. HORMONES AND MAMMARY TUMORS

As already pointed out, the apparent similarity in structure between the carcinogens, benzopyrene, dibenzanthracene, etc., and estrogenic substances such as estrone and estradiol, causes considerable uneasiness among certain sections of biochemists and clinicians. It must, however, be remembered that from the chemical point of view the relationship is not so close as the actual structural formulae would suggest. As pointed out by the writer in 1935 (2) there is much closer similarity between male and female sex hormones chemically than there is between estrone and, let us say, dibenzanthracene. Yet the clinician who is worried about the possibility of a carcinogenic action of estrone is not at all concerned about the possibilities of producing masculinization by giving a woman estrone. As already pointed out, estrone is not carcinogenic in the same sense as the condensed carbon ring carcinogens. It has been shown, however by Lacassagne (24) and his co-workers that mammary carcinoma can be induced in certain strains of mice, provided they are given very large doses of estrone over a prolonged period. Since the original observation, this whole subject has been thoroughly explored by an extensive series of researches. A full review of these has been given by Lacassagne (25). Briefly, it would appear that estrogenic stimulation will increase the incidence of mammary carcinoma in the mouse, provided the strain is one possessing a spontaneous incidence. By prolonged estrone treatment the incidence can be considerably raised. There appears to be no doubt today that the action is due, not to the direct carcinogenic action of estrone, but to its estrogenic or gynecogenic activity. This is proved by the fact that similar results may be obtained when estrone is replaced by the synthetic analogues stilbestrol and hexestrol. These substances do not possess the condensed carbon ring system. Hence, the activity in this respect must be due entirely to their estrogenic properties.

One is tempted to speculate whether the work of Bittner (1) on mammary cancer transmitted through the milk could be considered under the heading of a hormonal reaction. This is not the place to summarize this most important branch of cancer research. Those wishing to obtain a short summary of the position up to date should refer to the excellent leading article in a recent British Medical Journal (29). The evidence up to the present would seem to indicate that the agent which passes into milk, and causes a high incidence of mammary cancer in the offspring, is probably not present in an ordinary simple solution. Bittner in his observations on high-speed centrifugation inclines to the view that the factor is of high molecular weight, and is probably present in colloidal form. The facts at present would fit in with the virus conception.

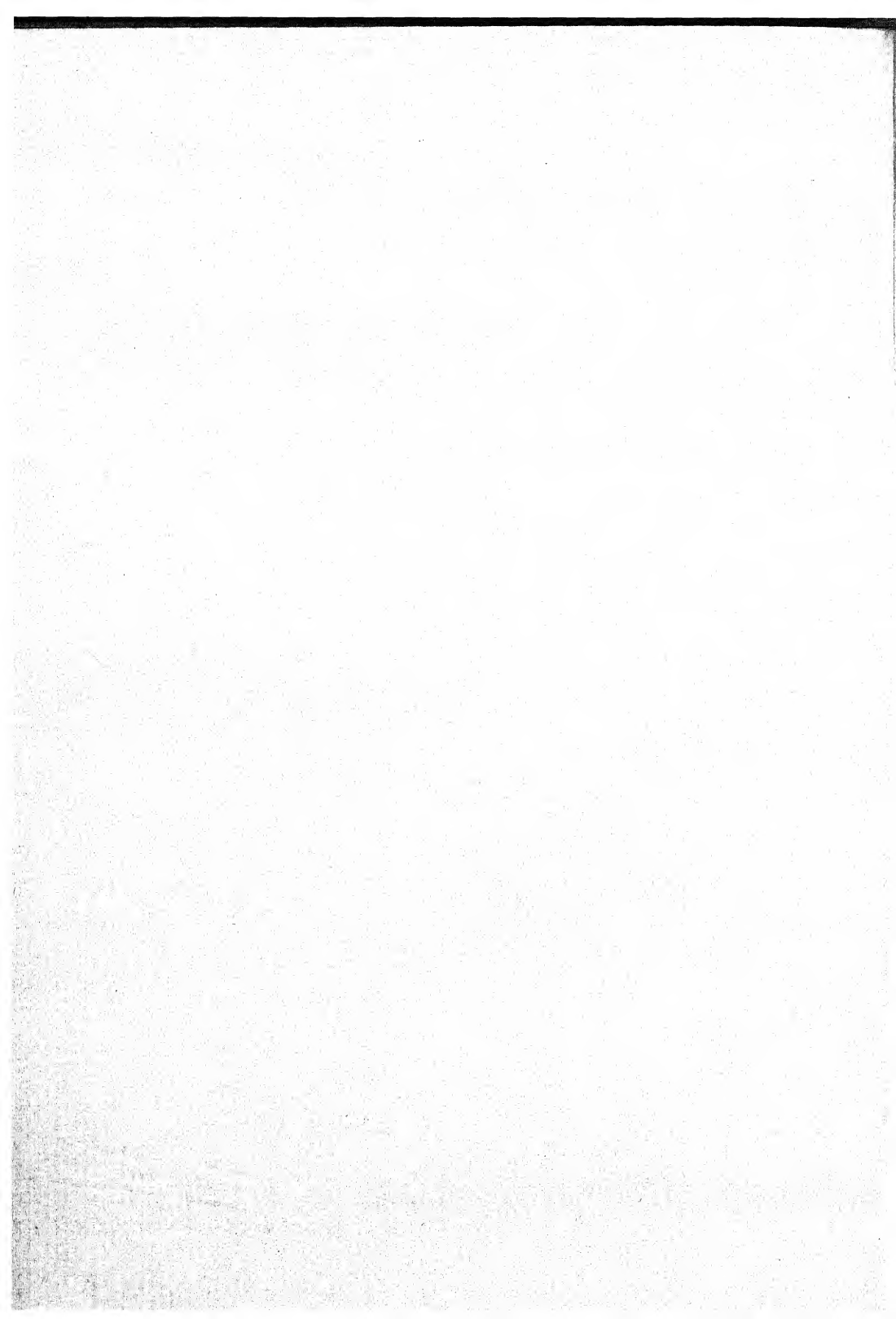
There must also be mentioned here the literature on the administration

of hormones for growth inhibition. A large number of publications has appeared very recently on this subject. The problem bristles with experimental difficulties, since there are so many factors influencing the rate of growth of tumors. Thus, as pointed out in the introduction, a substance may be toxic to the animal and damage its health to such an extent that the tumor growth is impaired. As at present the results are so doubtful, it would seem better not to attempt an accurate summary of the position until further information is available.

In conclusion it would seem that by far the most hopeful indications of the important rôle being played by hormones in the cancer problem are the reported effects of estrogens.

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Effect of Androgens and Estrogens on Birds¹

By A. S. PARKES AND C. W. EMMENS

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INTRODUCTION

This review deals with the effects of androgens and estrogens on the reproductive organs and secondary sexual characters of birds, and is restricted to a discussion of the effects of crystalline, characterized substances. The older work, dealing with extracts of various body fluids and

¹ Some of the material in this review has been taken from a manuscript entitled "Effects of Estrogens and Androgens on the Male" prepared by us early in 1939 for Volume III of *Ergebnisse der Vitamin- und Hormonforschung*. The outbreak of war made it impossible to return the corrected proof to Germany and, so far as we have been able to find out, the article was never published.

tissues, has been reviewed adequately by previous writers (*cf.* for instance, Allen, 3).

The nomenclature used for the androgens is that derived from the early suggestions of Butenandt and of Ruzicka, and now widely accepted. The nomenclature of the estrogens is that in general use, based originally on the suggestions of Adam, *et al.* (1), and afterwards accepted and extended by the European representatives at the Second Conference on the Standardization of Hormones, 1935, and by the Council on Pharmacy and Chemistry of the American Medical Association.

A. Effect of Androgenic Substances

I. DESCRIPTION OF SUBSTANCES

The chemistry of the androgens is outside the scope of this article, and reviews by Ruzicka (189), Koch (146, 147), Butenandt (18), Dannenbaum (56), Fieser (94), and Goldberg (115) should be consulted for details.²

Three of the commonly used androgenic substances are known to occur naturally; these are androsterone, dehydroandrosterone, and testosterone. Certain other androgens isolated from natural sources, such as adreno-sterone (Reichstein, 182), androstadienone (Burrows, Cook, Roe, and Warren, 16), and isoandrosterone (Butler and Marrian, 30; Pearlman, 175) are of much less importance in a consideration of the biological properties of this group of substances. A large number of compounds closely related to androsterone and testosterone, and showing androgenic activity, have been artificially prepared. The simplest members of the series differ from each other only in the permutations of keto- and hydroxy- groups in positions 3 and 17, in the spatial configuration of the hydroxyl group, and in the presence or absence of an unsaturated linkage in Ring A. The chief saturated members of the series (Fig. 1) have all been extensively investigated, and a great deal is now known of their biological activity. They are:

Androsterone	(Androstane-3- <i>cis</i> -ol-17-one)
Isoandrosterone	(Androstane-3- <i>trans</i> -ol-17-one)
Androstanediol	(Androstane-3- <i>cis</i> -17- <i>trans</i> -diol)
Androstanedione	(Androstane-3:17-dione)
Dihydrotestosterone	(Androstane-17- <i>trans</i> -ol-3-one)

Other saturated compounds, including *trans*-androstanediol (androsterone-3-*trans*-17-*trans*-diol), androstane-3-*cis*-17-*cis*-diol, and androstane-17-*cis*-ol-3-one, are of little importance.

² See also G. Pincus and W. H. Pearlman, *Vitamins and Hormones* vol. I, 293 (1943); T. Reichstein and C. W. Shoppee, *ibid.*, page 345.

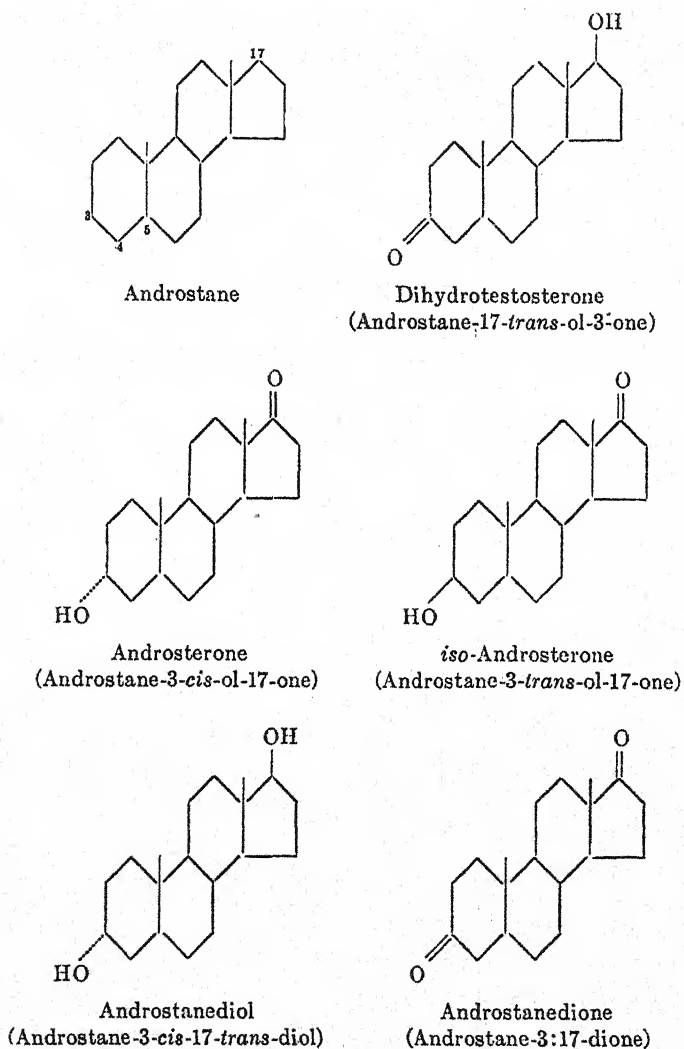


Fig. 1. Graphic Formulae of the Chief Saturated Androgens

Several unsaturated members (Fig. 2) of the series are well known. Those much used for experimental work (at one time or another) include:

- trans*-Dehydroandrosterone (Δ^5 -androstene-3-*trans*-ol-17-one)
- trans*-Androstenediol (Δ^5 -androstene-3-*trans*-17-*trans*-diol)
- Androstenedione (Δ^4 -androstene-3:17-dione)
- Testosterone (Δ^4 -androstene-17-*trans*-ol-3-one)

Among other unsaturated compounds which have been little investigated or are of slight biological importance are *cis*-dehydroandrosterone (Δ^5 -androstene-3-*cis*-ol-17-one), *cis*-testosterone (Δ^4 -androstene-17-*cis*-ol-3-one), *cis*-androstenediol (androstene-3-*cis*-17-*trans*-diol) and androstene-3-*trans*-17-*cis*-diol. Variants of certain of these compounds have been made by transference of the double bond to an abnormal position, *e.g.*

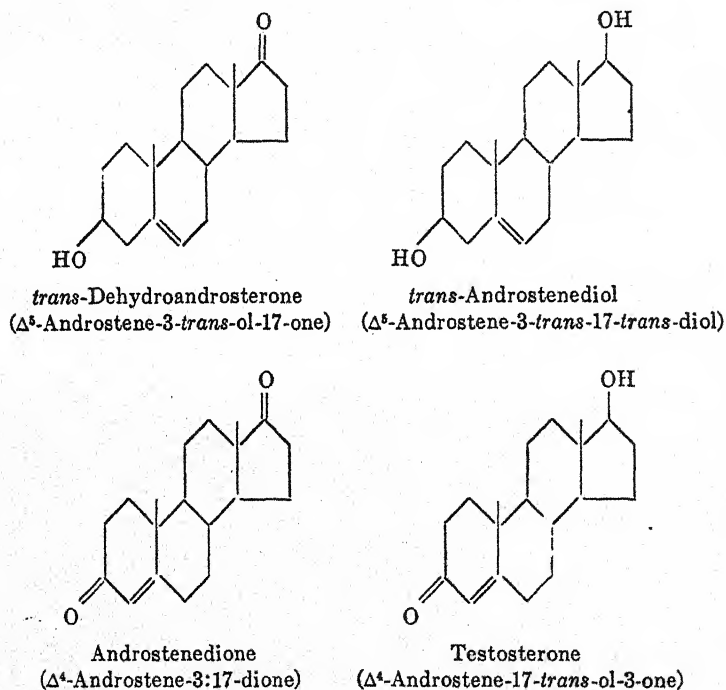


Fig. 2. Graphic Formulae of the Chief Unsaturated Androgens

Δ^4 -androstene-3-*trans*-17-*trans*-diol, Δ^4 -*trans*-dehydroandrosterone, and Δ^5 -androstenedione, but these again appear to be of little interest to the biologist.

A set of derivatives of members of this series has been obtained by alkylation in position 17; of these the methylated compounds have the greatest biological significance, though the ethylated ones are also important. Among the less important derivatives may be mentioned the oximes, which show comparatively little biological activity. Several other types of derivative have been made, such as 17-ethinyl- and 6-keto-testosterone, but they need not be considered here. Esterification of the compounds modifies their biological effectiveness considerably, and the long

series of aliphatic esters, and also the various benzoates, which have been produced are of great biological interest. Those most investigated include androsterone benzoate, androstanediol-3-benzoate, testosterone benzoate, and aliphatic esters of testosterone from the acetate to the valerate. Certain di-esters of the enolic form of testosterone have also been prepared.

II. EFFECT ON SEX DIFFERENTIATION OF THE EMBRYO

The developing bird embryo can very easily be subjected to the direct influence of exogenous hormones, and the peculiarities of gonad differentiation make such experiments of special interest. The embryonic gonad is morphologically indifferent until about the 6th day of development. In genetic males a pair of testes differentiate in the usual way, *vasa deferentia* develop from the Wolffian duct, and the Müllerian duct disappears between the 8th and 11th days. In genetic females, after the 6th day, an ovary differentiates from the left gonad, and an oviduct from the left Müllerian duct. The right gonad, on the other hand, remains rudimentary and indifferent in practically all species of birds, and the right Müllerian duct ceases to grow after the 8th day, and degenerates together with the Wolffian duct. At hatching, female chicks can easily be recognized macroscopically by the asymmetry of the gonads. Complete removal of the left gonad in the female, especially from the young chick, usually leads sooner or later to regeneration of the right gonad as an ovo-testis, which produces both androgens and gynecogens and causes growth of the comb as well as feminization of the plumage.

Successful attempts to modify the sex differentiation of the fowl embryo soon followed the production of crystalline androgens.

Wolff (207, 209) and Wolff and Wolff (215, 216) made a single injection of androsterone into the fowl's egg before the 10th day of incubation and produced various grades of intersexuality. Increasing dosage of the hormone diminished the number of both normal males and females hatched, until with a dose of 0.35–1 mg. only a few normal males and many intersexes were produced. The numerical results left no doubt that genetic males were affected, and the condition of intersexes confirmed the opinion that they were partly derived from genetic males. In Leghorns, larger doses were required to affect genetic males than were needed for the genetic females; in chicks of the cross between Light Sussex and Rhode Island Red the reverse seemed to be the case. The effect of androsterone on the male embryo was both androgenic and gynecogenic (Wolff, 209). There was genital hypertrophy, particularly of the right testis, which was not sensitive to gynecogenic action, while the left testis developed an ovarian cortex and became an ovo-testis. The proximal segments of the Müllerian ducts persisted as oviducts. Methyl-dihydrotestosterone had

the same action as androsterone but produced greater hypertrophy of the Wolffian duct. Testosterone acetate had little effect on the male embryos of the Light Sussex—Rhode Island Red cross in doses up to 1.0 mg., but had the same action as androsterone in Leghorns. Willier, Rawles, and Koch (201) stated that androsterone and dehydroandrosterone had feminizing and masculinizing effects on the bird embryo, while testosterone was only masculinizing. The first two stimulated development of ovarian cortex and persistence of the oviducts in genetic males, while in genetic females they inhibited the development of these components. Wolff (212) suggested that the anomalous effect of some of the androgens was due to their being substances with ambisexual activity.

Dantchakoff (59) called attention to the great hypertrophy of the mesonephric elements and the dilation of the Wolffian duct in chick embryos of both sexes when testosterone propionate was injected into the egg during incubation, a condition associated with a considerable development of the comb at hatching (Dantchakoff, 67) and with the premature appearance of song within ten days after hatching (Dantchakoff, 60). Androgenized chicks sometimes have bald heads at hatching (Dantchakoff, 63). Anomalies of the oviduct in pullets androgenized in embryonic life have been described by Wolff (211). According to Dantchakoff (62, 64) the genetically female gonad of the embryo chick is not inverted by male hormone, a fact which is discussed at length by the same author in another paper (Dantchakoff, 65). Gaarenstroom (107) found that the effect of androsterone and dihydroandrosterone (androstanediol) is much influenced by the time of injection. When introduced after two days' incubation, the substances may completely suppress the development of the Müllerian duct.

III. EFFECT ON FEMALES

1. *Secondary Sexual Characters*

Comb. The comb of the hen is larger and more vascular than that of the capon, and it is much reduced by ovariectomy until regeneration of the right gonad takes place. It follows, therefore, that the ovary of the hen produces some comb-growth promoting substance, probably a 17-keto-steroid, in addition to estrogens. In the circumstances, androsterone should cause growth of the hen's comb. This result was obtained by Callow and Parkes (32) who observed considerable enlargement in the comb of a Brown Leghorn hen injected daily with 2.5 mg. of androsterone (Fig. 3). Testosterone has the same effect (Emmens, 85).

Beak. Where the beak of the cock assumes a special color during the breeding season, under the influence of androgens, injection of androgens

will also cause the appearance of the characteristic coloration in the female. This reaction is well seen in the English sparrow in which the black coloration of the beak, normally seen only in the breeding male, can readily be induced in the normal or ovariectomized female by injection of androgenic substance (Witschi, 203). The Indigo Bunting and the African weaver finch, *Pyromelana franciscana*, as described by Witschi (204), also appear to be of this type. A rather different state of affairs is found in the female starling, in which injection of androgens, but not of estrogens, causes the appearance of the typical yellow coloration of the beak seen in both sexes in the breeding season (Witschi and Fugo, 205). As Witschi points out, this result shows that one of the normal secondary sexual characters of the female starling is androgen-conditioned. Though not strictly relevant to the subject of this review, attention may be called to

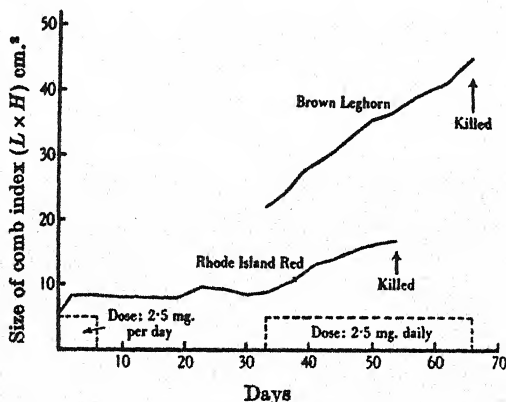


Fig. 3. Effect of Androsterone on the Size of the Hen's Comb (Callow and Parkes, 32.)

the very interesting observation of Pfeiffer and Kirschbaum (179). These authors found that the injection of comparatively large doses of gonadotrophic extract to the anestrus female sparrow led to over-stimulation of the ovary and to the appearance of the characteristic black pigmentation of the beak seen in the breeding male. This observation is most easily interpreted as meaning that under abnormal stimulation the ovary of the hen-sparrow may produce as effective amounts of androgen as the active testis.

Plumage. Androgens would not be expected to influence directly the plumage of normal females, but the case of the ovariectomized female is of some interest, since it has been suggested that the plumage of the hen shows a greater susceptibility to the action of estrogens than does that of the cock. If the ovary (left gonad) is removed from the female fowl,

the plumage changes to the neutral type, seen in the male, until the right gonad hypertrophies, when the plumage reverts to the female type. The work of Pézard (178), Domm (81) and others, shows that the plumage of the poularde is not feminized by grafted testis tissue and does not in this respect, therefore, differ from that of the caponized cock. In this connection it is relevant to cite Champy's observation (45) that large doses of testosterone will feminize the plumage of the ovariectomized female silver pheasant.

Voice. Hamilton and Golden (131) found that prolonged administration of testosterone propionate to female chicks or hens caused crowing and other male behavior. The injection of comparatively small amounts of testosterone over 10-15 days evokes typical male singing in female canaries (Leonard, 152; Shoemaker, 195; Voss, 199; Baldwin, Goldin, and Metfessel, 6).

Other Sexual Characters. In the immature or gonadectomized night heron (Noble and Wurm, 168) injection of testosterone causes the appearance of the coloration of the buccal cavity and legs seen in the female during the breeding season. Estrogens are without this effect, and it must be concluded, therefore, that some at least of the breeding season changes in the female night heron are androgen-conditioned (*cf.* beak coloration of female starling). On the other hand, administration of androgens also evokes male behavior, which is not seen, of course, in the normal female. Interesting observations on the psychical effects of androgenizing females have also been made in ring-doves by Bennett (8) who records that injection of testosterone to low-ranking submissive members of a flock of ring-doves of either sex resulted in an advance in status in the social hierarchy and increased aggressiveness. In addition, the females developed male behavior.

2. Ovary and Oviduct

Ovary. Herrick and Lockhart (133) found that a slight decrease in ovarian weight could be produced in the ovaries of young fowls by injection of testosterone.

Oviduct. Enlargement of the oviduct following the injection of androgens has been noted by Riddle (183) in the dove, and by Noble and Wurm (168) in the night heron. In the latter bird, however, there was no hyperplasia of the oviducal mucosa. Witschi and Fugo (205) noted in both the normal and gonadectomized female starling a similar oviducal response to androgens, which also caused hypertrophy of the Wolffian ducts.

IV. EFFECT ON INTACT MALES

The comb and wattles of the young cockerel can be made to develop rapidly by the administration of androgenic extracts (Juhn, Gustavson, and

Gallagher, 141). Füssganger (104) showed that great development of the comb and wattle of the chick could be obtained, by the inunction technique, with relatively small amounts of crystalline androgen. Breneman (10) investigated the effects of androstenediol benzoate and testosterone on the comb and testes of White Leghorn chicks. Subcutaneous injection of 0.1 mg. per day of either substance for 5 days into 5 or 10 day-old chicks led to immediate cessation of testicular growth, which apparently lasted only during the period of injection. One-month-old chicks which had been treated with male hormone from the 5th to the 10th day after hatching had an average gonad weight 90% heavier than controls. Injections of pituitary hormone together with androstenediol benzoate were effective in maintaining testicular growth, showing that the latter acted by depressing the gonadotrophic activity of the pituitary gland. Considerable comb growth occurred in the experimental birds. Breneman (11) has also shown that in such intact chicks the comb-growth-promoting action of testosterone propionate is less than that of androstenediol benzoate, and that whereas divided doses of the former are more effective than a single dose, the latter is surprisingly effective when given in a single injection. Combs continued to grow more rapidly than normally for several weeks after the cessation of injections, and the author suggests that this is due not to slow absorption of retained hormone, but to increased secretion of androgenic substance by the bird, since such long continued growth did not occur in injected capon chicks. Crowing occurred in nearly all treated intact chicks, but not in the treated capons. Hamilton (129) also records the induction of precocious sex behavior (aggressiveness and crowing) in male White Leghorn chicks at 10 days old by the injection of testosterone propionate, and observed stunting of body growth. Interesting observations along these lines have also been reported by Noble and Zitrin (169). These authors found that male chicks injected with testosterone propionate exhibited all the sexual behavior patterns of the adult cock. Crowing commenced as early as the 4th day of age and treading on the 15th. The copulatory pattern was identical with that seen in the sexually mature bird. Growth of androgenized males was retarded and the stimulated comb regressed to a subnormal level after the cessation of injection. According to Kosin (150) injection of testosterone into small chicks causes growth of the genital eminence in both sexes.

The use of the chick, either male or female, for the assay of androgens by injection or inunction is dealt with on p. 378.

Morato-Manaro, Albrieux, and Buño (163) produced a comb growth of 99.3% (measured by area) in a 3-month old cockerel by the inunction of 12.5 mg. of androsterone in 10 daily doses. The testes of this bird were greatly atrophied and damaged, showing no spermatogenesis and tubules with only 2 or 3 rows of cells and an amorphous mass containing dead cells

in their lumina. Damage to the testes was also observed by Chu (48) in pigeons injected with testosterone, though similar treatment of hypophysectomized birds resulted in some degree of testicular maintenance. By contrast, Koch (149) found that prolonged administration of testicular hormone to Leghorn cocks increased their fertility.

The production of abnormal growth of the comb in adult cocks has proved difficult, as was found by the earlier workers with impure extracts. Greenwood and Blyth (122) were unable to induce growth of the comb of the adult sexually active male fowl by the injection of male hormone, even by intra-comb injections of 100 γ of androsterone per day for 10 days followed by 200 γ per day for 5 days. However, by implantation into the breast muscles of a 6 mg. tablet of testosterone propionate, we have been able to increase the size of the comb of a normal Silver-Black Rock cock beyond the normal genetic limit in this breed.

V. EFFECT ON CAPONS

Those external secondary sexual characters of birds which are dependent for their integrity on the presence of the testis have been much used for the study of androgenic activity. The special characters may include head furnishings, notably the comb and wattles, the color of the eye and of the beak, crowing, sexual behavior, and, rarely, plumage. The effects of castration on the characters most commonly used, and the biological activity of androgens in maintaining or restoring them in capons, is described below. The adaptation of these results to the assay of androgenic preparations is dealt with in Section VI.

1. *Secondary Sexual Characters*

Comb and Wattles. When the young male chick is caponized further growth of the comb, wattles, and ear lobes is only sufficient to keep pace with the general enlargement of the body and head (Pézar, 176, 177). The adult capon, therefore, has a rudimentary comb and wattles, which are pale and dry. It should be emphasized that the capon comb is entirely different from that of the female. When an adult cock is castrated, the comb and wattles shrink very considerably, but not quite to the level of those of a bird castrated as a chick, since there has been a previous development of sufficient fibrous tissue to prevent full atrophy.

The histology of the comb of the normal cock and of the capon in various experimental conditions has been dealt with in detail by Hardesty (132).

Restoration or partial restoration of the comb of the capon was the first technique employed for the demonstration of androgenic activity in extracts of testis and urine, and it now seems certain that all substances which can be shown by any method to have androgenic activity will cause growth of

the comb and wattles of the capon. The degree of growth usually required for quantitative work is comparatively small (see p. 376), but a study has also been made of the complete restoration of head furnishings. Callow and Parkes (32) found that the daily intramuscular administration of 2.5 mg. rising to 5.0 mg. of androsterone, produced a comb in a Leghorn capon at the end of 3 weeks which was almost normal in size and which two weeks later was indistinguishable from that of a normal cock (Fig. 4). Emmens (86) produced very rapid and extensive growth of the comb of the Leghorn capon by the intramuscular implantation of a compressed tablet of crystalline testosterone propionate (Fig. 5). The maximum growth rate, however, was not in excess of that observed by Callow and Parkes, so it is likely that such a growth rate approximates to the maximum possible for the capon comb. According to Caridroit and Régnier (40) a daily dose of 0.3–1.7 mg. of testosterone propionate is necessary to maintain comb size in castrated cocks.

Beak. In a few species of birds the beak color differs in the two sexes and the difference may either be permanent or due to the assumption of a special color by one sex during the breeding season. In some such cases removal of the testes causes reversion of the beak color to that found during anestrus or in the female; thus, castration of the house sparrow causes the black color of the beak to fade to brown, as seen in the female. Administration of androgenic extracts causes the beak of the normal anestrus male or of the castrated male to assume the black colour within about 10 days (Keck, 142). The amount of androgen required to give an obvious response is of the same order as that required in the capon comb test carried out by intramuscular injection (Witschi, 203). The same effect can also be obtained by unction of alcoholic solution of testosterone propionate to the bill (Kirschbaum and Pfeiffer, 143). Witschi and Woods (206) described the histological basis of the changes in the beak of the sparrow. The essential feature is the presence, in the proliferating deep layer of the epidermis, of melanophores which produce melanin only when male hormone is present.

In the black-headed gull the development of the usual crimson color of the male beak during the breeding season is inhibited by castration (van Oordt and Junge, 198). In the indigo bunting and the African weaver finch the change in the color of the beak during the breeding season is also prevented by castration, and is therefore a secondary sexual character (Witschi, 202), presumably dependent on the presence of androgens.

Plumage. The color or structure of the plumage of most common breeds of domestic fowl is not affected by castration, which only causes a lengthening of the saddle hackles and tail feathers. The display plumage of such birds as the male Leghorn is therefore independent of testicular

activity and represents the neutral type produced in the absence of the ovary (Pézard, 176; Goodale, 116). In mammals, some androgens, includ-

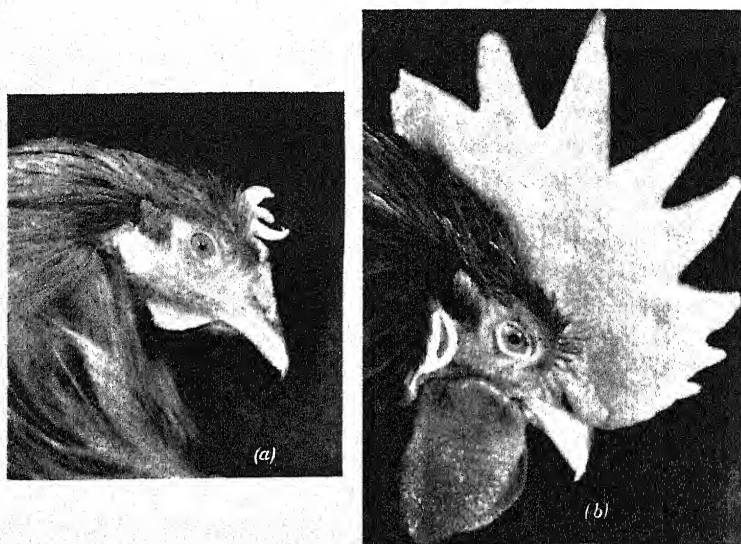


Fig. 4. Restoration of Capon Comb by Administration of Androsterone
(a) Before treatment.
(b) Same bird after receiving 22 daily injections of androsterone.
(Callow and Parkes, 32.)

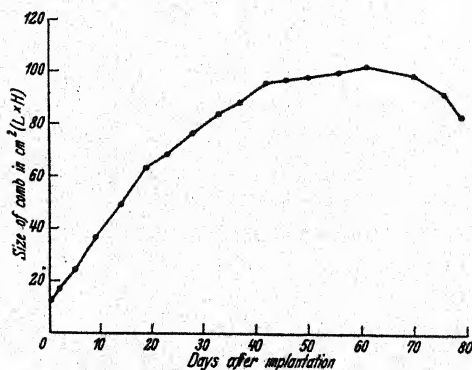


Fig. 5. Average Growth of the Comb of Eight Capons Each Receiving an Intramuscular Implantation of One Tablet of Testosterone Propionate, of Average Weight 11 mg.
(Modified from Emmens, 86.)

ing testosterone, exert a gynecogenic action such as causing enlargement of the uterus. Moreover, *trans*-androstenediol, if not other androgens,

possesses estrogenic properties, and will cause cornification of the vagina in ovariectomized mice. Nevertheless, it has not been found possible to demonstrate a feminizing effect of testosterone on the plumage of those breeds of fowl in which the cock normally has display ('male') plumage (Mühlbock, 1965). The only effect obtained by Emmens and Parkes (1952) on the breast feathers of the Brown Leghorn by massive dosage with testosterone and other androgens was similar to that which results from a temporary thyroid deficiency, and the absence of any feminization of the saddle feathers suggests that this is a likely explanation of the effects observed. According to Greenwood and Blyth (1954) it is possible to inject a sufficient amount of ground-up bird testis into a Brown Leghorn cock to cause apparent feminization of the plumage, but it is unlikely that this effect can be due to the presence of androgens.

There are, however, well-known instances of control of plumage characters by the testis. A type of considerable interest to the endocrinologist is that of the Sebright Bantam, where the plumage is the same in both sexes, that of the cock being of the female type. Gonadectomy of the cock causes change of the plumage to a type which would be expected in the male of such a breed. In the Sebright, therefore, the testis and ovary influence plumage in the same way, and direct changes are produced by androgens in the plumage of capons of such a breed. Deanesly and Parkes (1953) found that several of the androgens, possibly all of them in adequate dosage, will cause a change to female type in the feathering of the Sebright Bantam capon (Fig. 6). In addition, testosterone will cause similar changes, though less completely, in the plumage of the capons of other breeds such as the Campine, and certain game fowl varieties, in which the cock is henly feathered (Emmens and Parkes, 1952). It is probable, though not certain, therefore, that the capacity of the testis to feminize the plumage in such a breed as the Sebright Bantam is due to the fact that the plumage reacts to testosterone in the same way as it reacts to estrogens. If such a view is correct there is no longer any need to suppose, as was done by Callow and Parkes (1953), that the plumage of hen-feathered cocks is more sensitive to estrogens than that of normal breeds and can respond to the small amount of estrogenic activity known to occur in the testes in many species. An interesting case of testicular influence on plumage has been provided by Caridroit (1955) in extension of the work on the Sebright Bantams. When Silver Sebrights and Golden Sebrights are crossed, the male offspring are always silvered. These silvered cross-breeds become parti-colored after castration, but can be restored to full silver by injection of testosterone.

Another plumage type is seen in many species of duck in which dimorphism of the plumage occurs during the breeding season, and the drake, at the end of the season, acquires eclipse plumage which is superficially similar

to that of the female. The endocrine conditions upon which this change is based are obviously complex, and are not fully understood. Emmens and Parkes (92) were unable to affect the plumage of the capon mallard by administration of testosterone, but Chu (49) produced changes in the capon by the injection of dehydroandrosterone similar to those seen during eclipse in the normal drake. In other birds in which the plumage conditions are complex, such as the weaver finches described by Witschi, it is likely that the plumage is affected mainly by hypophyseal activity, and discussion of details in such cases is beyond the scope of this review.

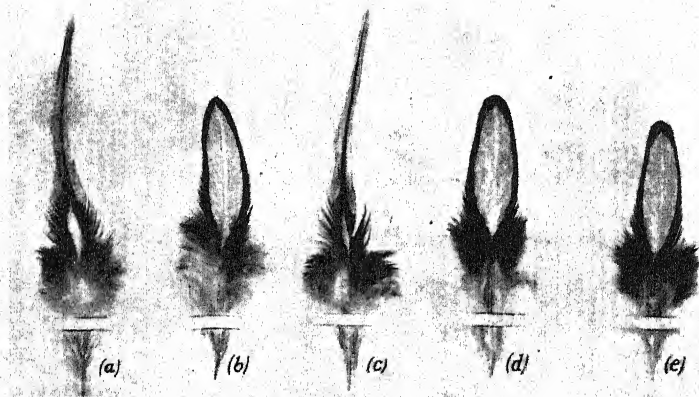


Fig. 6. Effect of Oestrone and Androgens on the Saddle Hackles of the Sebright Bantam Capon

(a) Normal capon saddle hackle.

(b), (c), (d), (e) Saddle hackles regenerating during the injection of (b) 5 γ oestrone daily, (c) 5 mg. androsterone daily, (d) 1 mg. *trans*-dehydroandrosterone daily, and (e) 1 mg. testosterone daily. (b), (d), and (e) are indistinguishable from the saddle hackles found in the normal

Sebright cock.

(Deanesly and Parkes, 73.)

One further small effect of androgens on the plumage of such birds as Brown Leghorns is seen in connection with the moult. The cock has an annual moult, while the capon tends to moult odd feathers during the whole year, and regeneration of feathers in denuded areas is also more regular in the capon than in the cock. Our own experience suggests that the administration of male hormone makes regeneration in the capon similar to that in the cock, and may interfere with the regularity of feather growth after plucking. Another minor point is that the administration of androgenic substances prevents the extra lengthening of the saddle hackles and tail feathering seen in the untreated capon.

Other Sexual Characters. It is well known that the capon, like a hen, does not crow, its voice being similar to that of the hen. This character is a good indication of the completeness of castration, since one of the first effects of testis regeneration is the onset of crowing. The effect of castration is not due to any change in structure of the syrinx, the voice box of the fowl (Myers, 1967; Appel, 4), and apparently it results from atrophy of the glottis, which determines the timbre, rhythm and modulation of the voice (Calvet, 34).

The cock's eye appears fiery, blood-shot and aggressive, characteristics which are not seen in the capon. According to Champy (41) the condition in the cock is due to convulsion and engorgement of the retinal capillaries,

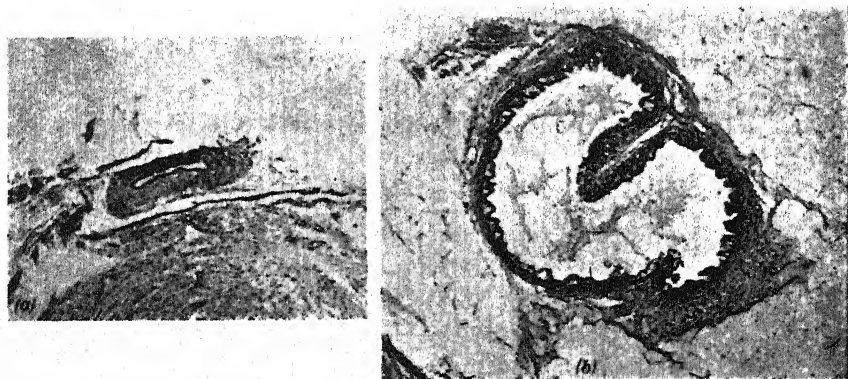


Fig. 7. Effect of Androsterone on the Atrophic *Vas Deferens* of the Capon

- (a) *Vas deferens* of untreated capon.
(b) *Vas deferens* of capon receiving 22 daily injections of 5 mg. of androsterone, showing restoration of epithelium and partial restoration of size.
(Callow and Parkes, 32.)

a condition which can be reproduced in the capon by the administration of androgens.

Sex behavior is absent from capons, and it disappears rapidly in cocks castrated when adult. Callow and Parkes (32) observed vigorous sexual behavior in a previously inert capon after 10 daily injections of 5 mg. of androsterone. Roussel (186) found that the sexual instincts returned to a capon after the daily administration of 1 mg. of testosterone acetate; crowing developed after 16 days and combativeness and treading after about 40 days, at which time the comb was approaching maximum size.

Castration does not affect spur development in cocks of normal breeds, but it allows spurs to appear in hen-feathered cocks. It does not seem to have been observed whether androgens suppress spur development in Sebright capons, as might be expected from their effect on plumage.

An interesting case of the dependence of secondary sexual characters on

androgenic activity has been reported by Noble and Wurm (168). In the black-crowned night heron there is a marked increase in the pigmentation of lore, lower mandible, and buccal cavity during the breeding season. There is also an intense vascularization of the legs, which take on a pink color. Castration suppresses the change in the male, whereas injection of testosterone initiates the changes in immature or castrated birds. Testosterone also induces adult behavior in chicks and causes hypertrophy of the male reproductive tract.

2. Accessory Organs

With the exception of *vasa deferentia*, accessory reproductive organs are comparatively ill-developed in male fowl, and one of the better known, the penis of the drake, is not very sensitive to castration (Champy, 42). A certain amount of work, however, has been done with the ducts. Callow and Parkes (32) were able to induce increase in size and epithelial development in the *vas deferens* of Brown Leghorn capons with androsterone (Fig. 7). They did not, however, obtain full restoration to the normal condition. Dessau and Freud (76) observed a similar result with testosterone.

VI. ASSAY AND RELATIVE ACTIVITY OF ANDROGENS ON BIRDS

1. Assay

Capon Comb. Gallagher and Koch (109, 110) were the first to devise a quantitative method for assaying androgenic extracts by measuring the growth of the capon comb. They defined a capon unit in terms of the average amount of growth in a group of birds, and established a 'characteristic curve' for extracts. Koch (146) reported that the same type of 'characteristic curve' in comb growth response holds for androsterone, *trans*-dehydroandrosterone, androstenedione and androstanediol. The shape of the dose-response 'curve' has been variously interpreted as a straight line when the dose is plotted logarithmically (Greenwood, Blyth, and Callow, 125; Hall and Dryden, 128), a parabola (Gallagher and Koch, 111) or a straight line with the dose plotted in ordinary units (Emmens, 89) (Fig. 8). Womack and Koch (217), Womack, Koch, Domm, and Juhn (218), and Koch and Gallagher (148) showed that the response of the capon comb is influenced by its initial length, and by the light intensity during the test. The temperature (Hain, 127) and the season of year (David, 69) at which the test is performed also influence the result. Moreover, capons of different breeds show very different sensitivities; in general, capons of light breeds are more sensitive than those of heavy breeds (Callow and Parkes, 32).

To increase the ease of housing the birds, Parkes (172) investigated the possibility of using capons of Bantam breeds, and concluded that Old

English Game Bantams become highly suitable capons, having an adult size of about 500 g.

Most of the authors cited above express comb growth in terms of the increase in length plus height obtained, but several other methods have been employed. Freud (100, 101) and Freud, de Fremery, and Laqueur (102) determine change in the area of the comb by direct photography of the silhouette of the comb before and after injection. Gradstein (117, 118),

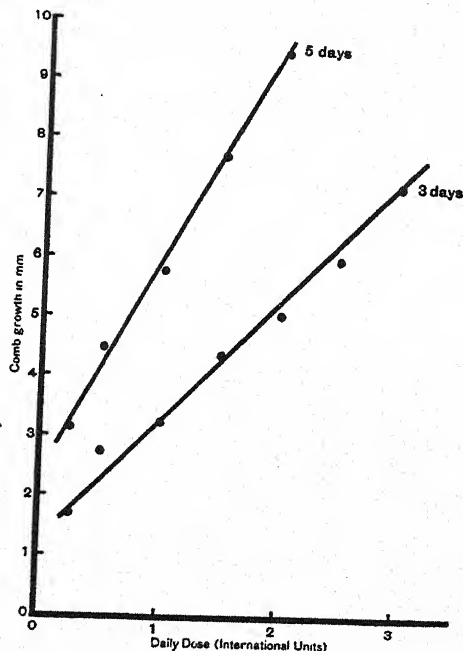


Fig. 8. Dose-Response Curves for International Standard Androsterone, Tested by Increase in Length Plus Height of the Comb of the Adult Brown Leghorn Capon

The upper curve is for the total dose given by injection over 5 days.
The lower curve is for the total dose given by injection over 3 days.
(Emmens, 89.)

Ogata and Ito (171), Ogata, Hirario, and Tonaka (170) use photo-electric or planimetric methods and also base their results on increase in area. Engel (93) investigated the possibility of determining the volume of the comb by measuring its displacement in water.

Tests are usually carried out by daily injection for 5 days, but results of equal value are obtained by giving the same total dose over 3 days (Emmens, 89). Since there is little lag in the appearance of the maximum effect when non-esterified substances are used, measurements are made on the day after the last injection.

Administration to the capon is most commonly made by intramuscular injection, but Greenwood, Blyth, and Callow (125) found that subcutaneous injection gave only slightly different results. Dessau and Freud (76) also found that the presence of impurities, notably fatty acids, in the preparation, which augment the action of testosterone in rats, do not have this effect in capons treated by intramuscular injection. The volume and nature of the solvent is also comparatively unimportant (Greenwood, Blyth, and Callow, 125). The capon test thus has the advantage that, provided injection is subcutaneous or intramuscular, the exact technique and the presence of impurities do not greatly influence the result. The use of other routes of administration may, however, greatly affect the result. Thus, local administration, direct application of the hormone solution to the comb by inunction (Füssganger, 104) or injection of a solution into the comb (Ruzicka and Tschopp, 191; Greenwood and Blyth, 122) leads to a much greater response per unit weight of hormone. Emmens (89) finds that the inunction of androsterone on to the combs of Brown Leghorn capons gives an approximately linear dose-response relationship over the range suitable for assay purposes. The response to direct inunction is much influenced by the vehicle in which the hormone is dissolved, alcohol, benzene, ether, arachis oil, and lanolin being decreasingly effective in that order (Emmens, 90).

Existing androgens are virtually inactive when given by mouth to capons (see Table III; and Kubo, 151), and oral administration is not therefore a practical method for assay purposes. Administration by intravenous injection is also inefficient since testosterone and even testosterone propionate are ineffective in doses of 1 mg. when given by this route in a single injection (Deanesly and Parkes, 72).

Chick Comb. The practical inconvenience of using fully grown capons for assay purposes has led to detailed examination of the possibilities of using young chicks, as suggested by Ruzicka (188). Burrows, Byerly, and Evans (17) investigated the effect of subcutaneous and intra-comb injections of androsterone and testosterone in White Leghorn and Rhode Island Red chicks of both sexes from 1 to 10 days old, and found that injection into the comb was a particularly sensitive method. Frank and Klempner (97) applied oily solutions of androsterone to the combs of 6-day old White Leghorn chicks, continuing daily treatment for 10 days, and produced curves showing the increase in comb weight over that of control groups obtained with varying total doses. Slight modifications of this technique were later introduced by Frank, Klempner, and Hollander (98). Danby (53) repeated these observations, and found that 100 γ of androsterone is approximately equal in action to 30 γ of testosterone in White Leghorn chicks. Attempts to work out a quantitative technique, based on subcu-

taneous injection of the hormone into chicks were also made by Dorfman and Greulich (83). Emmens (89) gives assay curves for both sexes in day-old Brown Leghorn chicks, using an inunction technique involving two applications with a 5-day interval between (Fig. 9). The two sexes give dose-response curves differing in slope and standard error, and on the basis of Emmens' results the use of sex-linked chicks would be desirable.

Examination of various methods suggested for employing chicks in the assay of androgenic activity was made by Duff and Darby (84), McCullagh and Guillet (156) and Hoskins, Beach, Coffmann, and Koch (135), all of whom concluded that it was extremely difficult to get quantitative results. These adverse conclusions were stoutly rejected by Frank, Hollander, and Klempner (96), and Frank, Klempner, Hollander, and Kriss (99) subsequently gave a detailed account of their technique. It is possible that a closer control of environmental conditions will give greater reliability to the test on the chick comb. For instance, Starkey, Grauer, and Saier (196) found that the amount of light affected the response, as do the nature and volume of the solvent (Klempner, Frank, and Hollander, 144; Klempner, Hollander, and Frank, 145). According to Breneman (13, 14) growth of the comb is produced more readily when the chicks are maintained on a restricted diet.

International Standard. To facilitate comparison of quantitative results obtained in different laboratories, and to supersede units defined in terms of bird reactions, the Second International Conference on the Standardization of Hormones, in 1935, established an international standard preparation for androgenic activity and defined a unit in terms thereof. This standard was composed of *crystalline androsterone* and the unit was defined as *the specific activity present in 100 γ of the preparation*. The Conference concluded on the evidence before them that the test based on the induction of growth in the comb of the capon by intramuscular injection was the only one sufficiently quantitative and specific to be recommended. The size of the unit thus defined is such that in the experience of most workers the injection of 1 International Unit (I.U.) daily for 5 days will give an easily measurable but not inconveniently large growth of the comb.

2. Relative Activity

Table I has been compiled from data given by Butenandt and Tscherning (28), Butenandt, Dannenbaum, Hanisch, and Kudzusz (22), Butenandt and Kudzusz (25), Butenandt and Hanisch (24), Butenandt, Tscherning, and Hanisch (29), Butenandt (19), Butenandt and Dannenbaum (21), Butenandt, Cobler, and Schmidt (20; cf. Koch, 146, 147), Tschopp (197) who summarizes the biological finding of the Ruzicka group, Deanesly and Parkes (70), and Bomskov (9). Compounds are listed in descending order

of activity and the amount in gamma equivalent to 100 γ (1 I.U.) of androsterone is given after each.

Only with three compounds do large discrepancies occur between the findings of different workers. In the list, the lower figures for *trans*-androstenediol and methyl-androstenediol were found by Deanesly and Parkes and the higher ones by Butenandt, *et al.* and by Tschopp, who gave the figure 500 for the non-methylated compound. Deanesly and Parkes give the higher figure for 17-methyl-testosterone and Tschopp the lower one.

TABLE I

Activity of Various Androgens in International Units (I.U.)

Compound See p. 000 for precise descriptions	Weight of I. U. γ
Testosterone.....	13-16
Methyl-dihydrotestosterone.....	15-24
Androstenediol.....	23-33
Dihydrotestosterone.....	25-30
Methyl-testosterone.....	25-70
Methyl-androstenediol.....	27-38
<i>cis</i> -Androstenediol.....	35
Ethyl-androstenediol.....	38
<i>cis</i> -Dehydroandrosterone.....	100
Androstenedione.....	100-120
Androstenedione.....	120-125
Δ^4 - <i>trans</i> -Androstenediol.....	150
Methyl- <i>trans</i> -androstenediol.....	155-525
<i>trans</i> -Dehydroandrosterone.....	200-300
Δ^4 - <i>trans</i> -Dehydroandrosterone.....	210
<i>cis</i> -Dihydrotestosterone.....	300
<i>trans</i> -Androstenediol.....	235-650
<i>cis</i> -Testosterone.....	500
Androstenediol (17- <i>cis</i>).....	350
Methyl- <i>trans</i> -androstenediol.....	500
Ethyl- <i>trans</i> -androstenediol.....	550
<i>trans</i> -Androstenediol.....	550-600
Isoandrosterone.....	500-770
<i>trans</i> -Androstenediol (17- <i>cis</i>).....	850-1000

In a redetermination of the activity of these three compounds we found the following figures:

<i>trans</i> -Androstenediol	400
Methyl- <i>trans</i> -androstenediol	140
Methyl-testosterone	66

The only significant change from Deanesly and Parkes' figures is that for *trans*-androstenediol.

The relation between chemical configuration and biological activity in androgens of the androstane series is fairly clear. In tests on producing comb growth in capons, when administration is by intramuscular injection, there is a marked superiority of compounds having a ketone or a *cis*-hydroxyl group in the 3-position over those with a 3-*trans*-hydroxyl group (e.g. androsterone and androstanedione compared with isoandrosterone; *cis*-androstanediol and dihydrotestosterone with *trans*-androstanediol). This superiority seems in general to be more marked in the saturated than in the unsaturated series, but it nevertheless holds in the latter. In the 17-position a *trans*-hydroxyl group is superior to a keto-group, which in turn is superior to a *cis*-hydroxyl group (e.g. androstane-3-*cis*-17-*trans*-diol, androsterone, and androstane-3-*cis*-17-*cis*-diol). This gradation is well seen both in the saturated and unsaturated series. Desaturation, either in the 4-5 or 5-6 position, does not markedly affect biological activity on capons except in the chemically abnormal compounds with Δ^4 - and a 3-*trans*-hydroxyl group, Δ^4 -*trans*-dehydroandrosterone and Δ^4 -*trans*-androstanediol, which are more active than the corresponding saturated compounds, and the also abnormal Δ^5 -androstenedione, which appears to be less active than the corresponding Δ^4 - or saturated compounds.

Effect of Method of Administration on Absolute and Relative Activity. Quite different values for the relative activity of some of these compounds have been found when they are given orally or by direct inunction on to the comb. Dessau (74, 75) reported that testosterone is only slightly more active than androsterone when assayed by inunction, and that androstenedione, androstanedione, and androstanediol have about the same activity, while dehydroandrosterone is rather less active. Breneman (12) found that androstanediol benzoate caused greater comb growth in the chick than testosterone and that both were more effective by subcutaneous than by intraperitoneal injection. In Table II the values we found for dosage by inunction and by mouth are compared with those given by the injection technique, androsterone being taken as 100 in each case.

As shown in Table III, the actual amounts required for assay by inunction are, of course, very much less than, and by mouth very much more than those used for injection (Emmens, 87).

3. Prolongation of Effect

Esterification. The effect of a single intramuscular injection of free hormone is transient, owing to the rapid absorption and utilization or destruction of the substance. Certain esters, however, have a prolonged action. Thus, Butenandt and Tscherning (27) noted that androsterone acetate had a somewhat prolonged effect, and Ruzicka, Goldberg, and Meyer (190) made a similar observation in the case of the benzoate. Callow (31)

recorded that the response to 5 mg. of androsterone benzoate given over 5 days was reached more than 20 days after the last injection. Miescher, Wettstein, and Tschopp (160) subsequently prepared a series of aliphatic esters of testosterone. They found that, when 6 daily doses of 50 γ were given to capons, the maximum response of the comb occurred 2 days after the last injection with the formate and acetate, 5 days after with the propionate, and 7 days after with the *n*-butyrate (Fig. 10). When a dose required to induce about a 30% maximum increase in comb-size was given, the maximum occurred on the day of the last injection with the free hor-

TABLE II

Comparative Amounts in μ g. of some Androgens Required to Equal the Activity of 100 μ g. of Androsterone when Administered by Different Routes

Substance	By injection	By inunction	By mouth
Androsterone.....	100	100	100
Testosterone.....	16	70	56
Androstanediol.....	33	80	556
<i>trans</i> -Dehydroandrosterone.....	300	200	500
Methyl-testosterone.....	66	150	73

TABLE III

Approximate Amounts (μ g.) of Various Androgens Required to Produce a 5 mm. Increase in Length plus Height of the Combs of Brown Leghorn Capons when Given in Oily Solution by Different Routes

Substance	Assay method			Dose by mouth
	Inunction	Injection	By mouth	Dose by injection
Androsterone.....	3.0	500	4500	9
Testosterone.....	2.1	85	2500	30
Androstanediol.....	2.4	165	25000	150
<i>trans</i> -Dehydroandrosterone.....	6.0	1500	22500	15
Methyltestosterone.....	4.5	330	3300	10

none, 1 day after with the formate and acetate, 3 days after with the propionate, 6 days after with the *n*-butyrate, 7 days after with the isobutyrate, 8 days after with the isovalerate, and 10 days after with the *n*-decanoate (Fig. 11).

The enolic diesters of testosterone are in general less intense but more prolonged in action than the monoesters (Miescher, Fischer, and Tschopp, 158). Thus, the maximum effect occurs on the 5th day after the last injection with the 3,17-diacetate; on the 7th day with the 3-acetate-17-propionate; on the 9th day with the 3,17-dipropionate, and on the 10th day with the 3-acetate-17-*n*-butyrate. Deanesly and Parkes (71) found that

testosterone diacetate had actions on capons similar to those of the propionate. Roussel, Gley, and Paulin (187) injected 100 γ daily of several testosterone esters for two months, and found that the combs of birds treated with the propionate grew both faster and for a longer time than those of birds receiving other esters. The isovalerianate and *n*-valerianate had about the same activity, producing throughout the experiment about two-

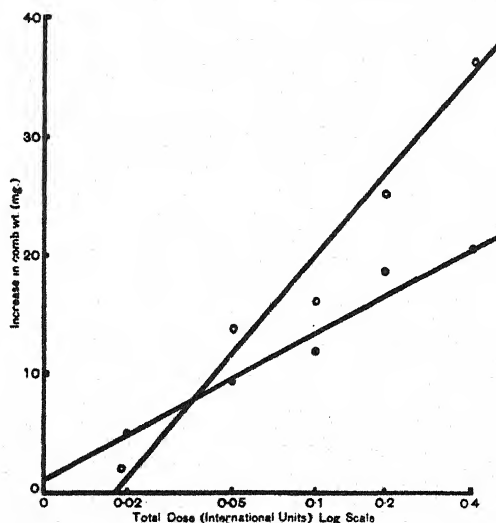


Fig. 9 (top left). Dose-Response Curves for International Standard Androsterone Tested by the Increase in Weight of the Combs of 10-day Old Chicks, Following Injections on the 1st and 5th Days.

○ = males; ● = females.
(Modified from Emmens, 89.)

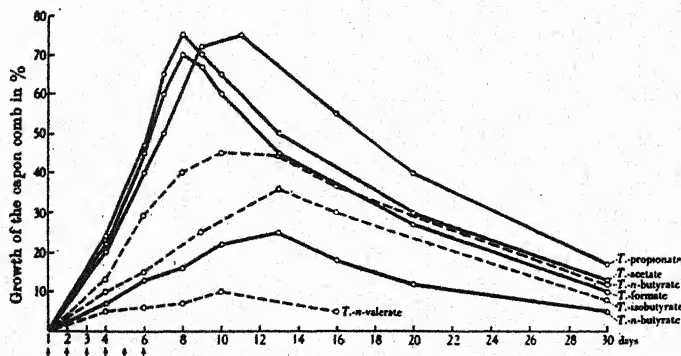


Fig. 10 (top, right). Duration of Effect on the Capon Comb of Esters of Testosterone

Six daily doses of 50 γ (—); Six daily doses of 100 γ (-----).
(Miescher, Wettstein and Tschopp, 160.)

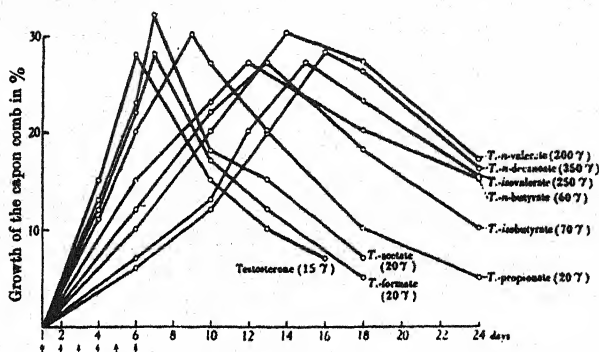


Fig. 11 (below, left). Time of Maximum Response of the Capon Comb to Various Esters of Testosterone Given in Doses Required to Produce About 30% Increase in Comb Area
(Miescher, Wettstein, and Tschopp, 160.)

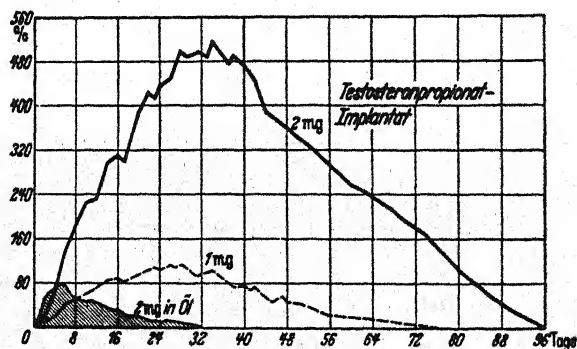


Fig. 12 (below, right). Relative Effectiveness of Testosterone Propionate in Restoring the Atrophic Comb of the Capon, Given by Injection in Oil Solution and by Tablet Implantation
(Schoeller and Gehrke, 193.)

thirds of the growth found with the propionate, but the acetate and trimethyl acetate did not produce further growth after about the first 20 days.

Aliphatic esters of androsterone and androstenediol have been considered in some detail by Schoeller and Gehrke (193). This prolongation of effect by esterification is not found following intravenous injection and is therefore due to delay of absorption from the site of injection (Deanesly and Parkes, 72). With some of the higher esters of testosterone, the palmitate and stearate, and with benzoates of androstenediol and androstenediol, the action may be so protracted that the intensity is too low, with ordinary doses, to produce an appreciable effect.

Implantation of Crystals or Tablets. Very high effectiveness and prolongation of action can be obtained in capons when androgens are adminis-

tered by the intramuscular implantation of solid crystals or tablets of the pure hormone (see p. 371 and Figure 5). Thus, Schoeller and Gehrke (193) found that 2 mg. of testosterone administered in this form produced a maximum response of 480% increase in comb size 16-21 days after the implantation, whereas an equal amount given in oil solution gave a comparatively negligible response of about 15% very soon after injection (Fig. 12). 2 mg. of implanted testosterone propionate gave a maximum response of about 500% increase 28-40 days after implantation. Schoeller and Gehrke obtained similar though less striking results with crystals of androsterone, androstenediol, and androstenediol. Emmens (86) investigated the maximum growth rate of the capon comb by the implantation method; giving tablets of testosterone propionate of 5-16 mg. per bird he was able to obtain maximum growth at 40-50 days, by which time the combs were the same size as in the normal cock, having increased 700-1000% in length times height. The average growth curve of the combs is shown in Fig. 5. Hamilton and Dorfman (130) found that crystals of testosterone propionate, implanted into day-old chicks, caused growth of the comb for 71 days.

B. Effect of Estrogenic Substances

I. DESCRIPTION OF SUBSTANCES

The chief naturally-occurring estrogens to be considered are (a) estrone, first isolated from the urine of pregnant women and later from the urine of stallions and pregnant mares, (b) estriol, from the urine of pregnant women, and (c) estradiol, originally prepared by hydrogenation of estrone, but afterwards isolated from the ovary itself by Doisy and his co-workers (Fig. 13). A full account of these substances, with references, is given by Marrian (155). As suspected by Schwenk and Hildebrandt (194), estradiol occurs in two forms owing to stereoisomerism at the 17-position; α -estradiol, the form found in the ovary, is considerably more active biologically than β -estradiol, which, in addition to the α -form, is found in pregnant mares' urine. The proportions in which the two are produced by reduction of estrone depends on the method employed. Two other estrogenic substances, equilin and equilin, have been isolated from the urine of pregnant mares by Girard and his co-workers (112, 113), but they have been comparatively little used for physiological work.

Estrogenic substances not so far known to occur in nature fall into two groups, (a) artificially prepared derivatives of members of the estrone series, and (b) certain synthetic substances, the more active of which are much simpler than estrone in chemical constitution (Fig. 13). The former, apart from the esters, have so far been of comparatively little biological importance, but ethinyl-estradiol is of special interest because of its high estrogenic activity by mouth. The synthetic estrogens have become of the greatest

importance since the observation by Cook, Dodds, Hewett, and Lawson (51) that estrogenic activity was shown by 1-keto-1,2,3,4-tetrahydrophe-nanthrene nucleus. This discovery dispelled the idea that such activity was specific for members of the oestrone series. Later it was found that even the phenanthrene nucleus was not necessary (Dodds and Lawson, 79). Subsequently, very highly active estrogens containing two benzene rings (Fig. 13) were prepared synthetically by Dodds and his collaborators. Of these, diethylstilbestrol (4,4'-dihydroxy- α,β -diethylstilbene) (Dodds, Goldberg, Lawson, and Robinson, 77) and hexestrol (4,4'-dihydroxy- α,δ -diphenyl-*n*-hexane) (Campbell, Dodds, and Lawson, 35) have been most extensively investigated.

Discussion of the assay of these various estrogens on ovariectomized female mammals is outside the scope of this review, but some reference to their relative activity is necessary. The international standard of estrone, by definition, has 10,000,000 I.U. per g.; estriol is considerably less active in the usual type of test, but strict comparison is impossible because the ratio found between the two substances depends largely on the exact technique of assay, as was shown in Section A VI (b) for the androgens. Curtis and Doisy (52) using a multiple injection technique found estrone to be about twice as active as estriol; Butenandt and Stormer (26) using a one-injection technique found it to be as much as 100 times more effective. Meyer, Miller, and Cartland (157) found it to be between 4 and 250 times as effective, according to the test method. Emmens (89) observed that estrone may be 2-3 times as active as estriol when compared by a 4-dose method or 70 times as active compared by a 2-dose method, both with oil solutions.

The early preparations of estradiol, containing mostly α -estradiol, were rather more than twice as effective as estrone (see for instance Schwenk and Hildebrandt, 194; David, 68). Subsequent work made possible the separate assay of the two forms. Butenandt and Goergens (23) found that α -estradiol was two and a half times as active, and β -estradiol only one-tenth as active as estrone. Hirschmann and Wintersteiner (134) reported that α -estradiol was twelve times as active as estrone, whereas β -estradiol was only one-twelfth as active as estrone. A detailed comparison by Emmens (89) showed that α -estradiol was about four times as active as estrone and that the ratio was not much influenced by the degree of subdivision of the dose.

Diethylstilbestrol was found by Dodds, Goldberg, Lawson, and Robinson (78) to be about four times as active as estrone in a 6-injection vaginal cornification test on rats, and to be about equal to it in activity by Emmens (87) in a 2-injection test with mice. Hexestrol is very similar to diethylstilbestrol in activity, though the exact relationship depends on the nature of the test and the technique of administration (36). Both are strikingly

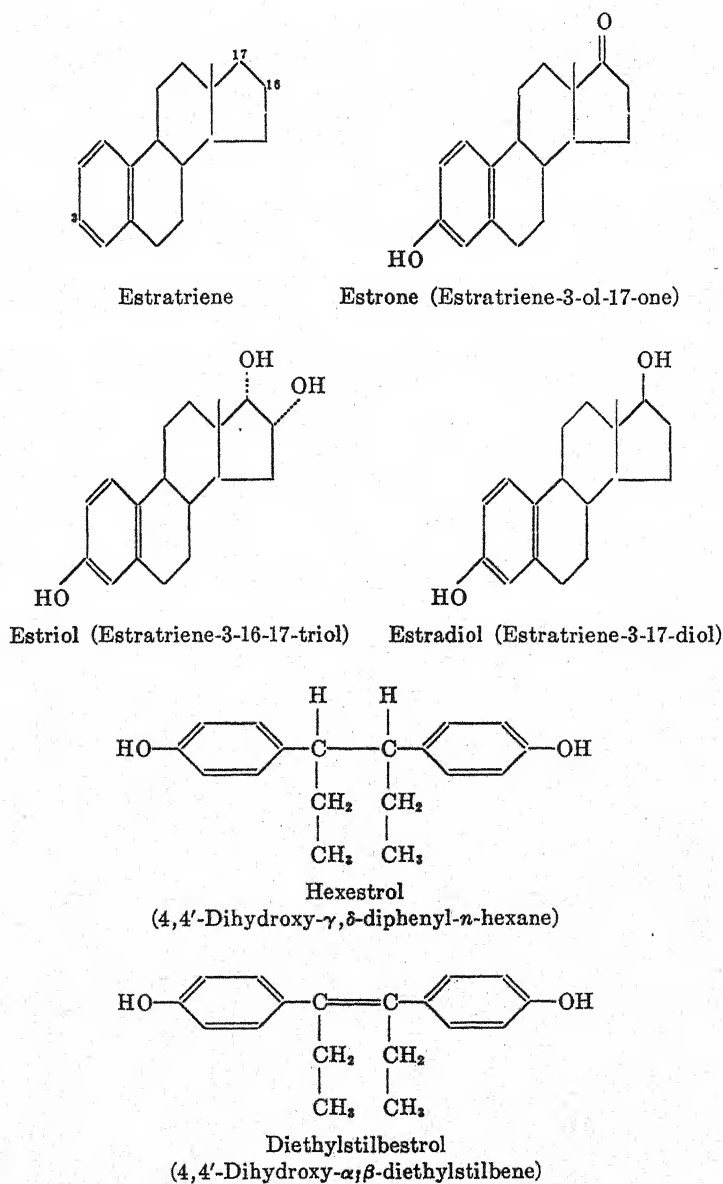


Fig. 13. Graphic Formulae of the Chief Naturally-Occurring and Synthetic Estrogens

different from the naturally-occurring estrogens in showing the same order of activity by oral as by parenteral administration.

II. EFFECT ON SEX DIFFERENTIATION OF THE EMBRYO

Wolff and Ginglinger (214) investigated the effects of estrone when injected into the chorio-allantoic membrane of the developing hen's egg, on or after the 5th day of incubation. Injection of 25 I.U. of estrone caused feminization of some males and was the minimal active dose. A dose of at least 100 I.U. was found to be effective in transforming all genetic males to intersexes, and one of 500 I.U. produced as strong a feminization as any higher dose. The intersexes formed an almost continuous series, but were divided for descriptive purposes into four types. The weakest intersexes



Fig. 14. Effect of Estrone on the Differentiation of the Gonad of the Chick Testis (at hatching) of chick which received 27 i.u. oestrone on the 5th day of incubation, showing embryonic testis tubules in the medulla with a cortex of ovarian tissue.

(From the material of Wolff and Ginglinger, 214.)

showed asymmetry of the gonads, the right gonad remaining a testis, while the left became an ovotestis, with an ovarian cortex external to a testicular core (Fig. 14). The strongest ones had a much reduced right testis and a normal appearing ovary in place of the left testis. The left Müllerian duct in the extreme type persisted as an almost typically female oviduct, while the right duct generally persisted longer than that of a normal female. These results were obtained with injections given up to the 8th day of incubation, after which even massive doses produced little effect, sensitivity being completely lost after the 12th day. Multiple injections were not found to increase effectiveness. According to Dantchakoff (57) the effect of estrogen is greatest if it is injected on the 4th day of incubation, less

during the 6th-8th days, and slight on the 10th day. Estradiol benzoate was also used in the later work (Wolff, 208, 210) and seems to have been less effective, weight for weight, than estrone, although it produced full intersexuality in doses of 1250 I.U. Diethylstilbestrol is effective in doses of 0.01 to 0.5 mg. (Wolff, 213).

Willier, Gallagher, and Koch (200) gave a single injection of up to 20 mg. of estrone or estriol 48 hours from the commencement of incubation, and found that the left gonad of genetic males had a central core of medullary tissue with a thick ovarian cortex. With the higher doses, the left gonad was entirely ovarian, in which case the right gonad also might contain ovarian medullary cords and be reduced in size. The ovaries of genetic females were not affected.

Gaarenstroom (105) has also shown that 100 I.U. of estrone influences the development of embryos up to the 6th day, producing intersexes and reducing the number of males to less than a quarter of the total hatch. The left gonads of "males" were found to be testes, ovaries, or ovotestes, and of intersexes predominantly ovaries or ovotestes. After more detailed work Gaarenstroom (107) reported that the feminization of the gonads of the genetic male embryo brought about by treatment with 0.1 mg. of estradiol benzoate or more is accompanied by the persistence of the left Müllerian duct. Large amounts of the hormone result in persistence of the duct in both sexes. He also found (106) that diethylstilbestrol produced similar results.

Riddle and Dunham (184) obtained interesting results on doves by injecting the hen 26-34 hrs. before ovulation was due. When estradiol benzoate (0.5-1.0 mg.) was given at this time most of the genetic males (sex-linked color) obtained from the eggs were intersexual. The intersexes were characterized by the left gonads being ovotestes, some of which were much larger and flatter than normal testes, and by the persistence of large Müllerian ducts. Genetic females remained normal. The authors point out that the appearance of spontaneous intersexes among the offspring of doves subjected to excessive egg production may be due to the presence, under these conditions, of excessive endogenous estrogens.

According to Dantchakoff (61) estrogenization of the embryo leads to some difficulties at hatching time, but the chicks are vigorous. As the modified chicks grow up (Wolff, 208) they lose the appearance of intersexuality and resemble normal males externally, the left gonad also developing more the histological appearance of a testis. Dantchakoff (62) also records this, but remarks that the oviduct and ovary-like appearance of the testis persist, although the plumage and head furnishings are male.

Greenwood and Blyth (123) noted that pullets derived from eggs injected with estrone at an early stage of incubation possessed two incompletely

developed oviducts and laid shell-less eggs. According to Dantchakoff (66) genetically female fowl treated with estrone during embryonic development are capable of normal reproduction when they become adult, whereas males are impotent but not aspermatic. Domm (82) records that adult males estrogenized during embryonic life showed a variable degree of feminization of the reproductive organs, especially of the left testis, but, after the first month, the plumage was usually of the male type. Dantchakoff (58) records the persistence of oviducts in such a bird.

Gaarenstroom (108) has also made interesting observations on the after-effects of estrogenization during embryonic life, and has produced clear evidence of the tendency of the birds to revert to normal as they grow up. Eggs of White Leghorns were injected on the 2nd day of incubation. The fowls produced were autopsied in three groups. At one week after hatching there were 80% of apparent females with ovaries and Müllerian ducts. At two months there were 50% females and 50% intersexes with ovotestes and greatly swollen Müllerian ducts, presumably derived from the genetic males. At nine months the sex-ratio was normal and in only two of the genetic males were ovarian follicles present in the testes, but the abnormal Müllerian ducts persisted in most of the males.

III. EFFECT ON FEMALES

1. *Secondary Sexual Characters*

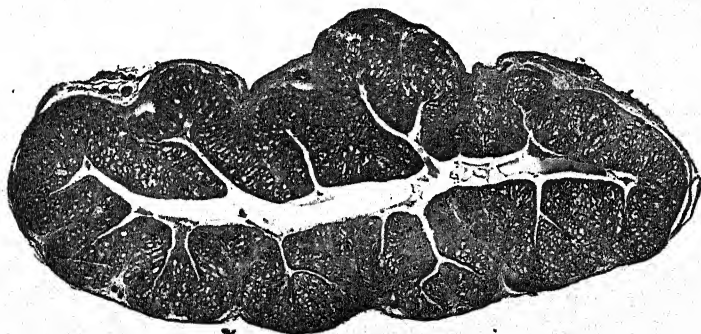
Comb. Régnier and Caridroit (181) found that weekly injections of 0.5-1 mg. of estradiol benzoate resulted in regression of the combs of growing hens. The effect was not found in adults during the laying season and was only slight during the rest of the year.

Beak. Two interesting examples of special beak color occurring in the female during the breeding season, and determined by the action of gynecogenic hormones have been described by Witschi (202). In the red-bill weaver finch the beak is brilliant-red in both sexes during anestrus or in castrates; in the breeding season the beak of the female, and of the female only, becomes yellow. This change can be reproduced in males, quiescent females, and castrates by injection of estrogens. Similarly, in love-birds (parakeets, budgerigars) the cere over the base of the beak is blue in the male, or in castrates, and brown in females. The brown coloration can be induced in males and castrates by injection of estrogens.

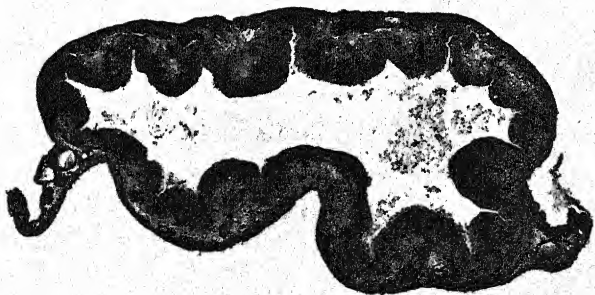
Sexual Receptivity. Noble and Zitrin (169) found that chicks injected with estradiol benzoate, starting on the 15th day of age, squatted for treading males after 18-26 days treatment. The behavior was typically that of a sexually receptive hen. Chicks in which the treatment was initiated on the 2nd day of life did not respond.



(a)



(b)



(c)

Fig. 15. Effect of Hormones on Oviduct of Immature Fowl
Oviducts of (a) untreated bird, (b) bird receiving 30 mg. of diethylstilbestrol over 2 weeks, and (c) bird receiving 300 mg. of testosterone over one week.
(All the same magnification.)

2. Oviduct and Crop-Gland

Oviduct. Early in the work on ovarian hormones it was shown by Riddle and Tange (185) that injection of estrogenic extracts of ovary or placenta would cause hypertrophy and hyperplasia of the oviduct of the immature pigeon. Similar results on fowl were recorded by Juhn and Gustavson (139) who also found (140) that the vestigial Müllerian duct of the male would respond to estrogens. Keck (142) found that estrogenic extract caused hypertrophy of the oviduct in anestrus or ovariectomized sparrows. Comparatively little work, however, seems to have been done with the crystalline estrogens. Wolff (210) caused great hypertrophy, accompanied by albumen secretion, in the oviduct of the young duct by the injection of large amounts, 12,000–25,000 I.U., of estradiol benzoate. Asmundson, Gunn, and Klose (5) found hypertrophy of the oviduct and hypoplasia of the bursa of Fabricius after injection of estrogens. Stilbestrol has the same effect in fowl (Fig. 15).

According to Noble and Wurm (168) both androgens and estrogens cause enlargement of the oviduct of the spayed night heron, but only estrogens cause hyperplasia of the mucosa. Riddle (183) found that some growth could be induced in the oviduct of the pigeon by injection of estradiol benzoate, but the largeness of the dose, and the smallness of the response led him to suppose that "relatively huge quantities" must be produced by the bird during the ordinary cycle. Chu (48) records that injection of estrone into hypophysectomized pigeons did not maintain gonadal activity, but hypertrophy of the oviduct was seen in the females.

Crop-gland. Folley (95) found that the simultaneous injection of estrone antagonizes the crop-gland stimulating effect of prolactin, and a maximum inhibition of 54% was observed. Similar results were reported by Bates, Riddle, and Lahr (7).

IV. EFFECT ON INTACT MALES

1. Plumage

The effect of estrogens on the plumage of cocks of ordinary breeds of fowl is essentially similar qualitatively to that seen in capons. Moreover, it is doubtful whether there is even any quantitative difference in the response of cocks and capons. The latter, however, are far easier to work with, and have usually been preferred as experimental animals. Most of the information about the effect of estrogens on plumage is therefore dealt with in Section B, V, b. Certain investigations have, however, been made on cock birds. Thus, Caridroit (37, 38) found that the plumage of the domestic cock could be completely feminized by the injection of estrone benzoate. This effect could be obtained at a dose level which did

not affect spermatogenesis or comb growth, or inhibit spur development. As in capons, feathers of different regions differ in sensitivity to estrogens, those of the dorso-lumbar region being most sensitive. Changes in feather form have a lower threshold than those in pigmentation. In breeds of fowl where the cocks are henney-feathered, injection of estrogen does not affect plumage. In a bird such as the pigeon, in which there is no sex dimorphism in plumage and no effect of gonadectomy, estrogens are also without effect on plumage (Lipschütz, 154).

2. *Testis and Comb*

The early post-hatching development of the testes and comb in chicks can be inhibited by the injection of estrone, as shown by Breneman (15). The same result can be obtained in partly grown fowl.

In more recent work, Morato-Manaro, Albrieux, and Buño (163), using White Leghorn cockerels three months old, applied 1.875 mg. of estradiol benzoate per day for 10 days, by inunction. Treated combs showed a reduction of 39%, and were white and floppy with less elastic tissue and more connective tissue than normal. The testes showed atrophy of tubules and interstitial tissue, and a sharp decrease in spermatogenesis caused, in the author's view, by depression of pituitary activity. The hind half of a comb divided by cautery, the fore half only of which was treated with 75 mg. of estrone over 18 days, showed some decrease in size.

Morato-Manaro, Albrieux, and Buño (162) described the effect of prolonged treatment with estrogens on the comb and testes of normal cocks and on the comb of the capon. They reported shrinkage and a change in histological appearance, similar to that which occurs in the capon, in the combs of cocks treated with amounts up to 50,000 I.U. of estrone in oil solution, injected over about a week. The combs decreased in size by 30%. The testes were pale and smaller than normal and showed reduced spermatogenesis and interstitial tissue. Zondek (219) recorded the production of 'pituitary dwarf' fowls, following the injection of 50,000-100,000 I.U. of estradiol benzoate twice weekly from the age of 6 weeks. The dwarf cocks were just over half the weight of normals, and showed spongy bones with open epiphyses, shrivelled combs, and atrophic testes reduced to 3.4% of the normal weight, and containing only Sertoli cells and spermatogonia. Emmens (85) administered large doses (up to 10,000 I.U. per week) of estradiol benzoate to adult Brown Leghorn cocks, and produced testicular atrophy, and comb atrophy almost as great as that seen in the capon (Fig. 16), and also inactivity of the thyroid epithelium with colloid-filled vesicles. The combs of these birds grew rapidly to a normal size after the injections were stopped. Further data (Emmens, 88) show that the implantation of 40-100 mg. tablets of estrone greatly reduces the size of the testes but

may hardly affect the comb. The condition of the testes of the birds treated with estradiol was as described by Zondek, and was somewhat similar to that seen in the hypophysectomized bird (Fig. 17). The testes regained an almost normal histological appearance, although still below normal weight, in a bird which was allowed to recover for 80 days after treatment. Comparable results in short experiments were obtained by Hoskins and Koch (136). Régnier (180) found that injection of estrogens depressed the growth of cock combs before or after puberty in the same way as castration. These effects are mainly to be ascribed to depression of pituitary activity by the massive dosage with estrogens, but it is probable



Fig. 16. Effect of Estrone on the Cock's Comb

(a) Before treatment.

(b) Same bird 37 days after implantation of a 65 mg. tablet of estrone.
(Emmens, 88.)

that the effects on the comb are also contributed to by direct local inhibition of the endogenous androgens by the exogenous estrogens. Such an effect would be in keeping with the known fact that the effectiveness of androgens on the capon comb can be decreased by the simultaneous administration of estrogen (see p. 403) and with the results of Morato-Manaro and Albrieux's (161) interesting experiments on divided combs. The probability of such a direct action is increased by Mühlbock's (164) demonstration that a very rapid shrinkage of the cock's comb can be obtained by the local application of estradiol benzoate. A partial depression of the activity of the anterior lobe is nevertheless clearly brought about by estrogenic treatment; Zondek (219) considers that only the growth and gonadotrophic

principles are seriously affected, though the cortico trophic and anti-insulin functions may be slightly depressed. Albrieux, *et al.* (2) showed that progesterone has no effect on the capon comb.

V. EFFECT ON CAPONS

1. Head Furnishings

It is generally agreed that the estrogenic substances have no stimulating action on the atrophic comb of the capon. Even when comparatively large amounts are tested by the sensitive technique of direct inunction, no comb growth-promoting activity has been found in estrone or estradiol (174). Moreover, Mühlbock (164) found that continued administration of high doses of estrogens caused further atrophy of the comb even in capons.

2. Plumage

In some birds, even where there is sex dimorphism in the plumage, the plumage type seems to be determined by genetic factors. Thus, the plumage of the English sparrow is affected neither by castration nor by ovariectomy; injection of androgens or estrogens is similarly ineffective (Keck, 142). In many species, however, hormones, notably estrogens, play a dominant rôle in determining plumage type. The fact that, in the fowl, ovariectomy leads to the appearance of display plumage, while castration does not affect the plumage type in ordinary breeds, indicates that ovarian hormone given to normal or castrated male birds might lead to feminizing of the plumage. Thus in Brown Leghorns, in which the breast feathers of the cock are black and those of the hen are fawn, the administration of estrogen to a capon or cock with growing feathers should cause them to take on the brown color seen in the female.

Freud, de Jongh, and Laqueur (103) were able to realize this theoretical expectation, and subsequently a great deal of work has been done on the feminization of plumage by the injection of estrogens, based mainly on the work of Juhn and Gustavson (139), Juhn, Faulkner, and Gustavson (138), Greenwood and Blyth (119), Champy and Demay (46, 47). The type of response obtained is greatly influenced by the growth-rates of different feathers, and of different parts of the same feather. The problem has been worked out in great detail by the Chicago school, including Lillie and Juhn (153). The chief conclusions from this work bearing on the response of feathers to ovarian hormone, are as follows: (1) A different rate of growth is shown by the feathers in different plumage tracts, and there is a correlated difference in sensitivity to estrogenic substance. For instance, the breast feather grows much more rapidly than the saddle-hackle, and is less sensitive to estrogen. (2) Not only is there a growth-

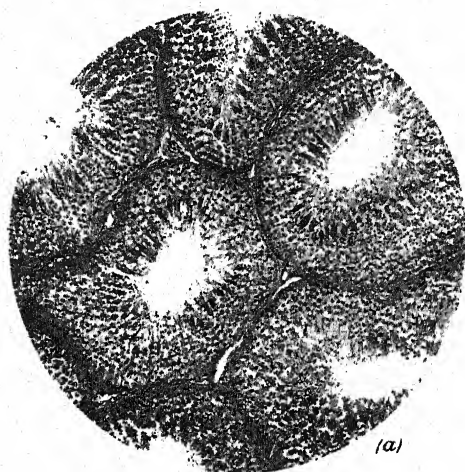


Fig. 17a

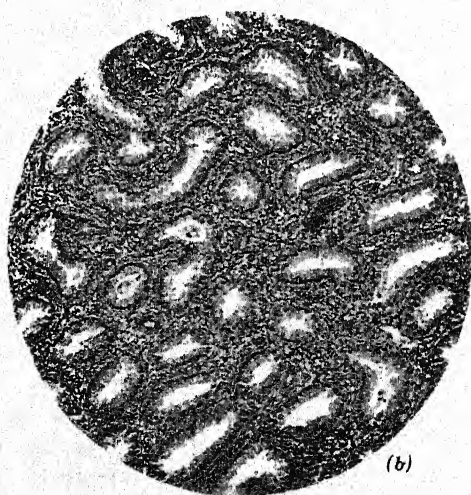


Fig. 17b

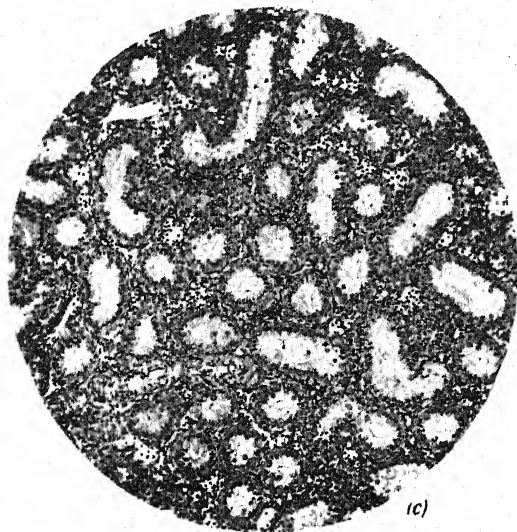


Fig. 17c

Fig. 17. Comparison of the Effect of Hypophysectomy and Estrogenization on the Testis of the Fowl

- (a) Testis of normal cock, showing active spermatogenesis.
- (b) Testis of cock 35 days after hypophysectomy, showing degeneration of tubules.
- (c) Testis of cock after administration of 105 mg. of estradiol monobenzoate by fortnightly and finally weekly injections over 110 days.
(Emmens, 88.)

rate gradient in different parts of the body, but gradients are exhibited by each individual feather. The growth-rate of the feather along its length, i.e. apical-basal growth-rate, is approximately constant, and the bottom of the feather has therefore much the same sensitivity to estrogen as the top. A sharp growth-rate gradient is, however, shown by the barbs, the tips of which grow faster than the bases. As a result, the base of the barb is more sensitive to estrogen than the tip, and it is possible to feminize the base with a level of estrogenic stimulation which is ineffective on the tip. This fact, together with the rapid response of the growing feather after injection of the estrogen and after it ceases to be available, makes it possible to get a great variety of response patterns. Some of the more extreme types found in the breast feathers of Brown Leghorn capons are shown in Fig. 18. A stimulus which is of short duration and only strong enough to affect the sensitive bases of the barbs results in a *rachis spot* (Fig. 18a). Such a stimulus is provided by a single small dose of a quickly absorbed estrogen. A stimulus of short duration but sufficient intensity to feminize any part of the barb results in a *narrow complete bar* (Fig. 18b). Such a stimulus is provided by a large dose of quickly available estrogen. A stimulus of low intensity exerted over a long time, such as is given by

the daily administration of small amounts of estrogen causes a *rachis stripe* (Fig. 18c). Finally, a strong stimulus applied for a long period, causes a *wide complete bar* (Fig. 18d). This type of stimulus is easily obtained by the daily injection of a large dose of estrogen. Application of such a stimulus during the whole time that a feather is growing will, of course, lead to the feather being entirely female in color. Analogous changes occur in feathers of other tracts, notably in the saddle hackles (Fig. 19). It should be emphasized that existing plumage cannot be altered, and an effect of circulating hormone is only visible when it acts on a feather in process of regeneration after a normal moult or after plucking an area. For experimental purposes the latter technique is adopted.

Greenwood and Blyth (120) made a careful study of the quantitative aspects of the response of Brown Leghorns. They found that neither the

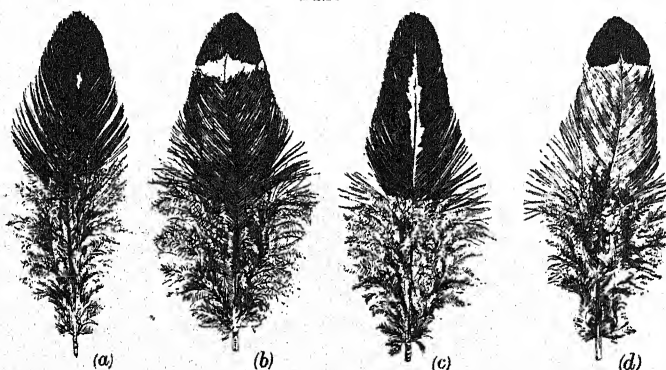


Fig. 18 (above). Types of Response of Breast Feather of Brown Leghorn Capon to Estrogenic Stimulation
Description in text.
(Parkes, 173.)

body weight of the bird, nor the season of the year, influenced the response, but the age of the bird was an important factor, because older birds responded more vigorously as determined by the amount of the feather feminized. They further made the most interesting observation (121) that intradermal injection of estrone produced an effect on feathers in the immediate locality and that a very small dose was effective. Although most of the work has been carried out on Brown Leghorns, enough is now known of other breeds to suggest that in every case in which the feathering of the hen is determined by the action of ovarian hormones, growing feathers in the cock or capon can be changed to the female type by administration of an adequate dose of estrogen. Thus, in the Sebright capon, the typical henny feathering can be restored by the injection of estrone (Champy and Demay, 47), while similar results have been obtained for

ducks, pheasants (Champy, 43, 44), and other birds (Emmens, 92). Different breeds and species, however, show very great differences in sensitivity to estrogens. Curious effects, indicative of genetic constitution, have been obtained by Danforth (55) and others, by the injection of estrogens into crosses between certain breeds of domestic fowl.

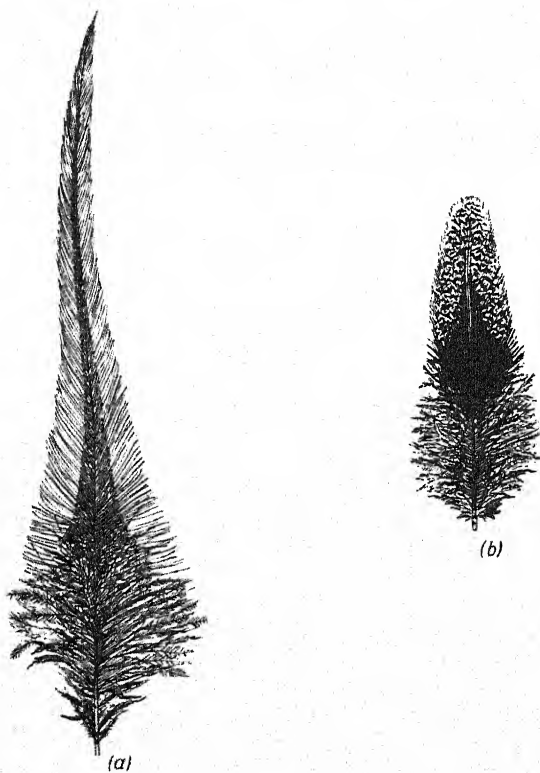


Fig. 19 (below). Effect of Estrone on the Structure of the Saddle Hackle of the Brown Leghorn Capon

(a) Normal saddle hackle.

(b) Saddle hackle regenerating in estrogenized bird, showing feminine structure.

All the estrogens which have been investigated, including the synthetic ones, seem to be able to evoke the plumage response. Thus, Cook, Dodds, and Greenwood (50) found that the response could be produced in Brown Leghorn capons by 1-keto-1, 2, 3, 4-tetrahydrophenanthrene and 9, 10-dihydroxy-9, 10-di-*n*-butyl-9, 10-dihydro-1, 2, 5, 6-dibenzanthracene. Diethylstilbestrol (Dodds, Lawson, and Noble, 80) and triphenylethylene (Hain, 126) have the same effect.

The rate of feather growth can easily be ascertained, so that the time during which the injection has yielded an effective concentration of hormone can be calculated from the width of the bar or the length of the stripe of female color. The feather reaction therefore offers an excellent test for the duration of action of estrogenic compounds when administered in various ways. In the strain of Brown Leghorns used by Parkes (173) the breast feathers of capons grew at the rate of about 2 mm. per day. Examination of the relation between dose of estrone, given as a single injection, and width of bar produced, showed that 0.1 mg. gave a faint response only, while 0.25 mg. and 1 mg. gave a response indicating effectiveness for about one day and two days respectively. Increase of dose above 1 mg. did not increase the duration of response. A similar result was obtained with estradiol, and it must be concluded that even large doses have only a transient effect.

The esters of estrone and estradiol, however, have a very different action. Estrone acetate is little different from the pure hormone, but estrone benzoate in a dose of 1 mg. produces feminization for 13 days. More recently, work on aliphatic esters of estrone, similar to those used by Miescher, Scholtz, and Tschopp (159) on female rats, shows that in birds also the prolongation of effect by esterification is proportional to the length of the acid chain. With the higher esters, however, the minimum dose required to produce any response is much increased. Parkes (173) also obtained similar results with esters of estradiol. The monobenzoate was slightly less effective than estrone benzoate; but the diesters of estradiol showed considerable extension of action over the corresponding mono-esters. Thus, a single injection of 1 mg. of the diacetate feminized the plumage for about 6 days, while estradiol-3-benzoate-17-acetate was found to be the most effective of the substances examined, the action of 0.25 mg. lasting for 11 days, and that of 1 mg. for 19 days. Estradiol dibenzoate has a very prolonged action, but the threshold dose is 100 times that of the free hormone. The mechanism of this effect of esterification was clearly shown by Deanesly and Parkes (72). These authors found that intravenous injection of free estradiol produced a response of the same duration as that following intramuscular injection (Fig. 20). The monobenzoate on the other hand, given intravenously, was much less effective than when given intramuscularly, being little better than the free hormone given intravenously (Fig. 21). The dibenzoate injected intravenously also produced a transient effect and had a threshold dose about the same as the free hormone. These results suggested strongly that the prolonged action by the esters was due to delayed absorption from the site of injection, not to delayed hydrolysis after absorption. This supposition was borne out by the further observations of Deanesly and Parkes that inunction

on to the surface of the skin just before the growing feathers emerged led to the effect of estradiol being as prolonged as that of the monobenzoate.

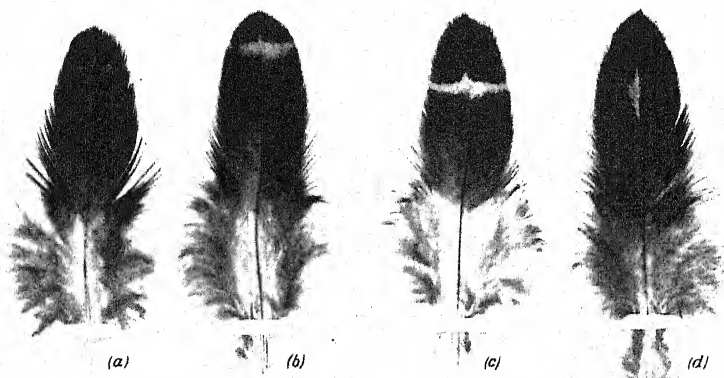


Fig. 20 (above). Response of the Breast Feathers of the Brown Leghorn Capon to a Single Injection of Free Estradiol Administered by Different Routes

- (a) 0.25 mg. in oil solution given intramuscularly.
- (b) 4 mg. in oil solution given intramuscularly.
- (c) 5 mg. in propylene glycol solution given intravenously.
- (d) 0.25 mg. in oil solution given by inunction on to the skin of the feather tract.

(Deanesly and Parkes, 72.)

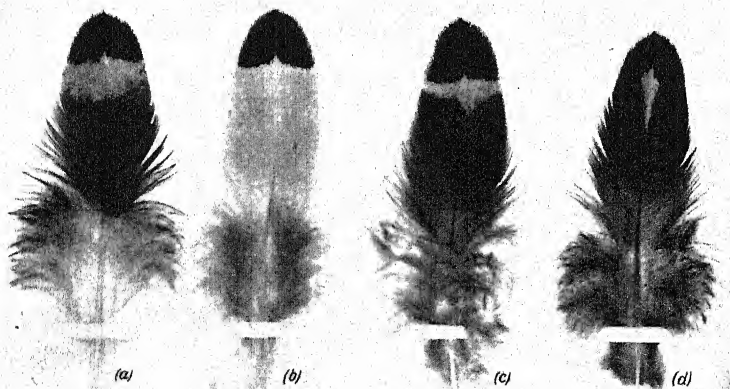


Fig. 21 (middle). Response of the Breast Feathers of the Brown Leghorn Capon to a Single Injection of Estradiol Monobenzoate Administered by Different Routes

- (a) 0.25 mg. in oil solution given intramuscularly.
- (b) 4 mg. in oil solution given intramuscularly.
- (c) 5 mg. in propylene glycol solution given intravenously.
- (d) 0.25 mg. in oil solution given by direct inunction on to the skin of the feather tract.

(Deanesly and Parkes, 72.)

The most conclusive result, however, was obtained by the intramuscular implantation of a 2 mg. crystal of estrone into each of a group of capons,

a treatment which feminized the plumage for nearly 3 months (Fig. 22), as compared with the 2-day result given by the same amount of hormone in oil solution. The nature of the effect of esterification in prolonging the response to intramuscular injection is thus amply shown by the fact that administration of the ester by a method which makes entry into the blood stream instantaneous (intravenous injection) removes its superiority, while administration of the free hormone in such a way that absorption is necessarily slow removes its inferiority.

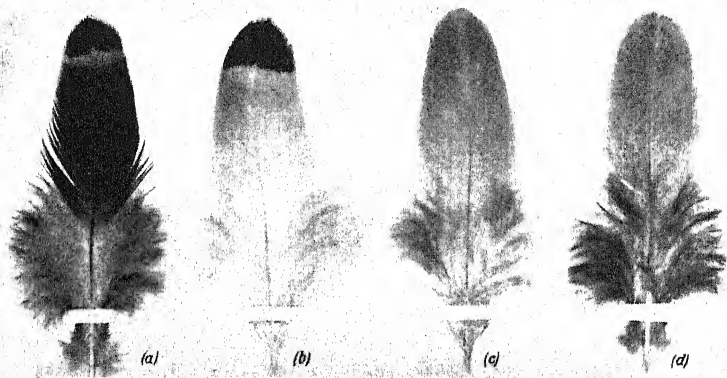


Fig. 22 (below). Response of the Breast Feathers of the Brown Leghorn Capon to Estrone

- (a) 4 mg. given in one injection in oil solution.
 - (b), (c), and (d) 3 mg. given by implantation of a solid crystal of pure hormone.
 - (b) Feather which had begun to grow at the time implantation was made.
 - (c) Feather which grew following a plucking 29 days after implantation.
 - (d) Feather which grew following a plucking 62 days after implantation. Reversion to the male type has begun at the base of the feather (d), so the total effect of the implantation of estrone has lasted for nearly 3 months.
- (Deanesly and Parkes, 72.)

VI. EFFECT ON RESPONSE TO EXOGENOUS ANDROGENS

Early in the work with active androgenic extracts, Juhn, d'Amour, and Womack (137) reported that the effect of androgenic or estrogenic extracts on capons was neither augmented nor inhibited by the simultaneous injection of the other. Schoeller and Gehrke (192) later found that the effect of male hormone concentrates on the capon comb might be increased by the simultaneous administration of estrogenic preparations. Workers using pure substances have been unable, however, to confirm this result. Callow and Parkes (32) recorded that 1 mg. of androsterone plus 1 mg. of estrone given over 5 days produced a slightly lower response than 1 mg. of androsterone alone. Morato-Manaro, Albrieux, and Buño (162) found that 30,000 I.U. of estrone inhibited the action of 0.15 units of male hormone when given with it as a mixed injection, and Morato-Manaro and Albrieux (161) afterwards reported further experiments in which

they found that the comb-growth-promoting effect of 2 'bird units' of androgen was completely inhibited by the addition of 25,000 I.U. of estrone. Gley and Delor (114) showed that estradiol benzoate decreases the response of the capon comb to testosterone, and Mühlbock (164) was able to abolish completely the response to 100 γ of androsterone daily by the simultaneous injection of 5 mg. of estradiol benzoate, or the inunction of 0.5 mg. of estrone or estriol daily (Fig. 23). Subsequently, Mühlbock (166) reported that when a technique of simultaneous unction with testosterone was used diethylstilbestrol did not exert the same antagonistic effect as estrone and estradiol. Emmens and Bradshaw (91), however, showed that this difference was purely quantitative and did not indicate any essential

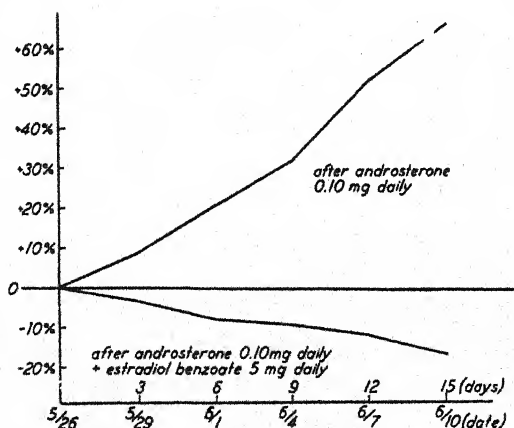


Fig. 23. Inhibition of the Effect of Androsterone on the Capon Comb by the Simultaneous Administration of Estradiol Benzoate (Mühlbock, 164.)

difference between diethylstilbestrol and the natural estrogens. Hoskins and Koch (136) found that the comb response in the capon to the administration of androsterone could be greatly reduced by the simultaneous administration of estrone. Emmens (88) obtained a progressive decrease in the response to testosterone propionate when increasingly large doses of estradiol benzoate were given simultaneously by injection at a different site. Even 10 mg. of the benzoate, however, did not abolish completely the response to 150 γ of testosterone propionate. Emmens also observed that the atrophic comb of the heavily estrogenized cock did not respond normally to 5 mg. of testosterone propionate. Danby (54) found that 2.5 mg. of estrone decreased the response of the chick's comb to 0.015 mg. of testosterone. There can thus be no doubt that estrogens have an antagonistic action towards the comb-growth-promoting properties of both testosterone and androsterone.

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X-Ray Crystallography and Sterol Structure

By DOROTHY CROWFOOT

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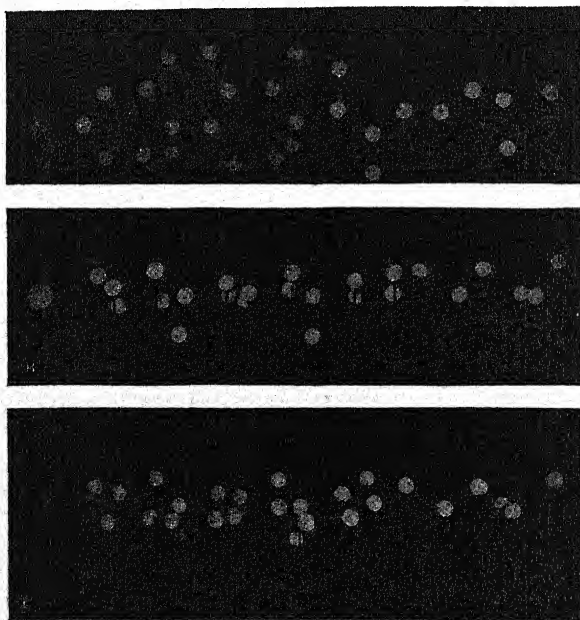
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I. INTRODUCTION

One of the most obvious properties of many naturally occurring sterols is their ready crystallization in characteristically similar crystalline forms. The appearance of cholesterol itself ready crystallized in gall stones naturally led to its early investigation—when Chevreul recorded its elementary analysis in 1823 he was already able to write a history of studies of its nature. And throughout the 19th Century we find records of crystallographic measurements combined with chemical research on sterols isolated from different plant and animal sources; even occasionally a crystal is described simply as of a "sterol." It is easy to see how crystallographic similarities arise now that we know, at least in outline, the structure of the molecules involved and the way in which these molecules must be packed within the crystals. It is easy also to see now why the investigation of sterol structure should have been a field in which X-ray crystallographic measurements could prove particularly effective.

It is the natural ambition of every X-ray crystallographer to determine in detail the exact atomic arrangement within the crystals under examination. In so doing he must of necessity also determine the chemical structure of the compound investigated, whether it be sodium chloride or a complex silicate, benzene or a sterol or protein. But as the compound becomes chemically more complicated, the interval before the final stage of the X-ray analysis is reached lengthens, and the intervening stages assume an importance of their own. This has been particularly true of the investigation of the sterols and of the groups of compounds related to them, bile acids, sex hormones, and heart poisons. There has in fact been an interval of ten years between the first X-ray measurements of J. D. Bernal on cholesterol, ergosterol, and calciferol (4), and C. H. Carlisle's detailed analysis

of one particular sterol derivative, cholesteryl iodide (19). The interval has, for historical reasons, been longer than would be necessary if the X-ray investigation of the sterols were to begin to-day since it has depended upon the development in the meantime of certain crystallographic techniques and the better understanding of others (41, 56, 60). But it has served to emphasize both the value of the information acquired in the early stages of



PHOTOGRAPH 1

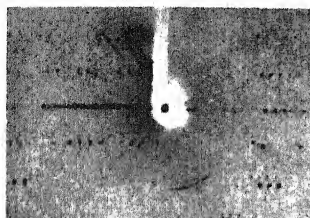
Model of Cholesteryl Iodide

A and B show views derived directly from the crystal structure (see Figs. 1-5). In C the photograph has been reversed to show a view more familiar to the chemist.

an X-ray investigation (25, 13) and also the necessity for the final complete analysis.

The X-ray investigation of the sterols was begun almost by accident at the suggestion of Professor J. B. S. Haldane in 1932 (4) and had as its object the determination of the position of calciferol in the main sterol series. This is an application of X-ray measurements which has recurred from time to time in this field. The X-ray diffraction pattern given by any crystal is usually very individual and the first use that may be made of it is as a means of rapid identification (13). But in addition to this in-

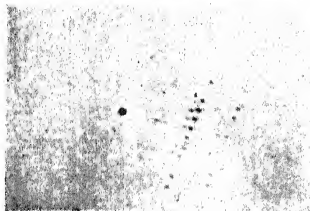
[100] || (001)



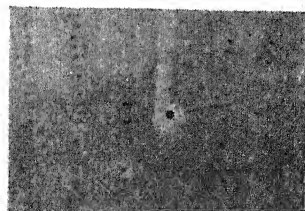
[100] || (010)



[001] || (100)



[001] || (010)



[010] || (001)

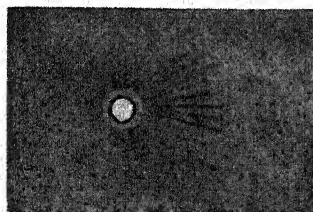


PHOTOGRAPH 2

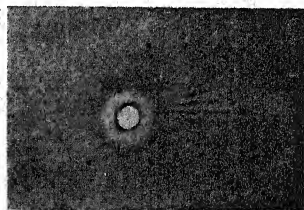
X-Ray Oscillation Photographs of Ergosterol

In each case the rotation axis is indicated followed by the beam direction at the beginning of the oscillation.

[001] || (100)



[001] || (100)



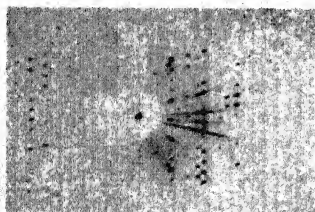
PHOTOGRAPH 3

X-Ray Oscillation Photographs of

a) γ -Sitosterol

b) Stigmasterol

\perp (001) || (100)



\perp (001) || (010)



PHOTOGRAPH 4

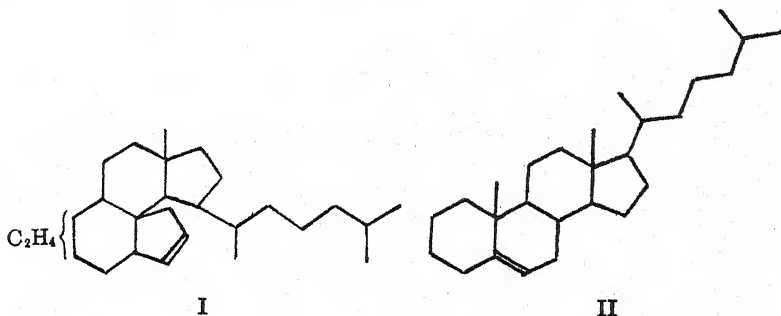
X-Ray Oscillation Photographs of Calciferol

dividuality there is often a general similarity in the crystal structures of compounds of closely related chemical structure. Certain of the sex hormone crystal structures—androsterone is an example—show a marked likeness to common types of sterol structure (5, 12). Most of the cardiac aglucone structures on the other hand are widely different; but here again it was possible to find a degradation product which from its X-ray photographs appeared closely related to the sterols (9). X-ray evidence was accordingly able to play a part in relating not only calciferol, but also these other groups, to the sterols.

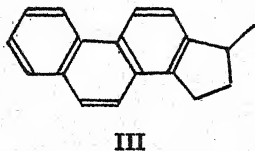
From the X-ray photographs obtained, the next step in the analysis is the determination of the size of the crystal unit cell and the space group or symmetry operations controlling the molecular arrangement. These, combined with the crystal density, provide a measure of the weight of the crystal asymmetric unit present. Three possibilities then arise: the crystallographic unit may be either equal to the chemical molecule or a multiple or a sub-multiple of it. The third case implies that the molecules have symmetry and does not operate here since all steroids are asymmetric. Usually the difference between the crystal unit and the molecule represented in the second case does not matter much; the order of magnitude of the molecular weight is known and, from the crystal data, a measurement can be made of its exact size, which is useful here in distinguishing the different molecular weight groups within the main sterol series *e.g.* cholesterol from ergosterol (13). Occasionally, when a new type of compound is first isolated, it is desirable to find a crystal in which the molecule is the unit, and this is usually possible by the examination of derivatives. Though calciferol itself happens to crystallize with four molecules in the asymmetric unit, the molecular compound pyrocalciferol-calciferol adopts an arrangement where there are only two, one of calciferol and one of pyrocalciferol. Calciferol therefore could be characterised crystallographically as a single chemical individual (4).

The unit cell dimensions, and the space group, themselves limit the possible shape and size of the molecule, but often this limitation is insufficient to be of much practical use. In addition, other evidence is desirable and in the study of the sterols the most valuable additional data were provided by the optical properties of the crystals, first examined by Bernal. The crystals of cholesterol, ergosterol, and calciferol were plates in which γ , the greatest refractive index, was inclined at a steep angle to the plane of the plate. The direction of γ adopted as that of the molecular length, together with β , the width, and α the thickness, gave, combined with the unit cell size, the characteristic dimensions of the sterol molecule, length 17 to 20 A.U., width 7.2 A.U. and thickness 5.0 A.U. These dimensions were quite incompatible (4, 6) with those required by the accepted Wieland-

Windaus 1928 sterol formula, I (72, 78). They led Rosenheim and King (63) to suggest the drastically revised version, which with the modification proposed by Wieland and Dane is that now accepted, II (7).



In the establishment of the details of this skeleton X-ray evidence was not at first able to play much part. There was a certain amount of work on degradation products of the sterols, particularly on Diel's hydrocarbon $C_{18}H_{16}$, III (27, 28), which was of assistance in showing the identity of the hydrocarbon obtained from the sterols with synthetic γ -methyl cyclopentano-phenanthrene (7, 11). Further, the close similarity in type of the crystal structures of the first sterols examined, particularly ergosterol, with the double layer structures of long chain alcohols indicated that the hydroxyl group in these sterols must be in a terminal position in the skeleton, *i.e.* in position C3 as finally adopted (4). Correspondingly the absence of a similar double layer structure among the sex hormones indicated that here the active groups were probably at opposite ends of the molecule (5). But in general so much chemical evidence was already available that the main outline of the sterol molecule was rapidly established without further assistance from X-ray data.



In greater detail, however, the structure of the individual steroids and even of their underlying skeleton present a number of problems, particu-

larly of stereochemistry, which are most difficult to solve by classical chemical methods. For the solution of these points the X-ray method is again available, but only if pushed to the final stage—the actual determination of the detailed crystal structure of at least one sterol derivative. The essential process involved in this determination is the calculation of the electron density in the crystal unit from the observed intensities of the X-ray reflections. But the process is not a direct one and its final success in the X-ray analysis of cholesteryl iodide (19) has depended on several favorable circumstances. In the first place, work on crystal structures in other fields had shown the simplifications introduced into the calculations by the presence of a heavy atom in the crystal (60). Secondly, methods had been developed for the ready calculation of the electron density in three dimensions, and these methods are essential in dealing with any non-planar molecule (40, 41). And thirdly, a wide survey of sterol crystals had been undertaken (13) which showed that, of the sterol compounds containing a heavy atom, cholesteryl iodide has a particularly favorable type of molecular arrangement on which to base an exact X-ray analysis.

In the analysis of cholesteryl iodide the position of every atom in this particular molecule has been fixed with a considerable degree of certainty. The arrangement found confirms the correctness of the skeleton (I) above, and also the correctness of the ideas about sterol structure expressed by Bernal as a result of the first X-ray measurements. The molecules are lath-shaped, and are packed roughly in the way originally suggested. The iodine atom is attached to C 3. Naturally a number of problems of sterol chemistry still remain to be solved, but the framework established for cholesteryl iodide must be present as a largely unchanged unit in the majority of sterol crystal structures. It seems, therefore, most worth while to review first in some detail the X-ray investigation of cholesteryl iodide, and then to discuss, with this as background, the remaining X-ray measurements in three of the main steroid groups, namely the sex hormones, heart poisons, and sterols. In addition two other crystallographic investigations have reached several stages towards completion and are of interest as providing data on contrasted chemical and crystallographic structure types. These are the analyses of palmitic acid-desoxycholic acid and of cholesteryl chloride and bromide. They too will form part of our background.

II. THE CRYSTAL STRUCTURE OF CHOLESTERYL IODIDE

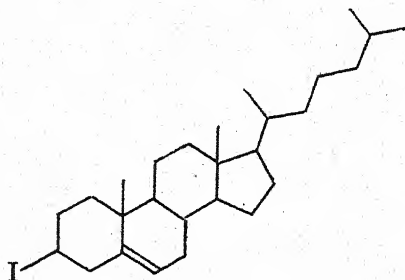
The aim underlying the X-ray analysis of cholesteryl iodide has been the determination of the structure of the molecule using as little chemical information as possible. As mentioned above, the data we have to work with are the observed intensities of the X-ray reflections, and the quantity which we wish to calculate is the electron density at every point in the

crystal unit cell, $\rho(xyz)$. The two are related by the formula first given by W. H. Bragg (15 cf. 16) involving the use of Fourier series:—

$$\rho_{xyz} = \sum_{-\infty}^{\infty} \sum_{-\infty}^{\infty} \sum_{-\infty}^{\infty} F_{hkl} \cos [2\pi (hx + ky + lz) - \alpha_{hkl}]$$

Here the quantity F_{hkl} , the structure factor or amplitude for a reflection from any particular plane (hkl) can be derived directly from the observed intensity of the reflection. But the second quantity, the phase angle, α_{hkl} , can only be said to be directly "observable" in particularly simple structures, where it is fixed on first principles from the symmetry and limited number of atoms present (*e.g.* in sodium chloride). In more complex cases two methods for "observing" phases have been attempted: (1) the examination of two isomorphous structures identical except for the replacement of one atomic species in the first by a heavier atom in the second (59, 21), and (2) the introduction of one outstandingly heavy atom whose contribution alone should largely determine the phases of the X-ray reflections (60).

The second method appeared to us much the most promising for our purpose. It has already been applied with success in several researches, the most notable of which is J. M. Robertson's analysis of platinum phthalocyanine (60). Here the platinum atom is at a centre of symmetry in the crystal structure, and makes a maximum contribution to every X-ray reflection. These contributions far outweigh those of all the carbon and nitrogen atoms in the structure, and hence the phase of every reflection is that of the platinum atoms alone. A Fourier series formed direct from the observed F values of the ($h0l$) reflections and using these phases showed a projection of the molecule in which the positions of all the carbon and nitrogen atoms present were clearly discernible.



IV

In cholesteryl iodide, IV, the situation is rather more complicated. Neither crystal nor molecule has a centre of symmetry. The atoms do not lie in a single plane, and hence X-ray analysis in three dimensions is essen-

tial. The positions of the heavy atoms are not fixed by crystal symmetry and hence have first to be found. And lastly, since the molecules are asymmetric, the positions of the heavy atoms in the crystal will be related to one another by higher symmetry than are the positions of the molecules as a whole. The phases deduced from the heavy atom contributions will in turn introduce greater symmetry than the true phases. And the use of these phases will therefore only limit the possible positions of the atoms in the structure and not fix them unambiguously in three dimensions as in the case of the phthalocyanines.

At the outset of the X-ray analysis of cholesteryl iodide an interesting complication occurred. A second crystalline modification was found in the preparation, very similar in cell dimensions to the first but differing in the intensities of the reflections. The unit cell dimensions are compared in Table I. Both structures appeared suitable for exact analysis. In both there are only two molecules in the unit cell, related to one another by a

TABLE I
Unit Cell Dimensions of the Two Forms of Cholesteryl Iodide

	<i>a</i>	<i>b</i>	<i>c</i>	β	ρ	<i>n</i>	Space Group
Cholesteryl Iodide A	10.93	10.34	21.47	149°	1.300	2	P2 ₁
Cholesteryl Iodide B	12.57	9.04	21.89	149°	1.275	2	P2 ₁

simple two-fold screw axis of symmetry (space group, P2₁). And the fact that the direction of this axis of symmetry, the crystallographic *b* axis, is also the direction of the least refractive index, α , of the crystals suggested that it was roughly normal, in both cases, to the plane of the sterol ring system. A detailed examination of both structures was therefore begun.

The first step in the structure analysis was the preparation of projections of the structure on a plane normal to the *b* axis. Crystallographically these particular projections should have a centre of symmetry, and, accordingly, the heavy atom phase angles in this plane should closely approximate to the true phase angles. The projections were easily obtained from the measured intensities of reflections of the type (*h*0*l*). The actual position of the iodine atoms in the projections were first deduced from vector maps prepared by Patterson's method using the observed F^2 values (56). For these projections the phases need not be known. The peaks appear at vector distances from the origin which correspond to prominent interatomic vectors in the structure. The iodine-iodine vector introduces such a much more powerful peak than any others that it is readily identified (Figs. 1a and 2a) and parameters assigned to the iodine atoms in the crystal. These are given in Table II. Fourier projections were then derived for both structures using the observed *F* values and the phase angles calculated from the

iodine contributions. These are actual projections of electron density, and are shown in Figs. 1b and 2b.

These two projections are very interesting. In both it is easy to detect the outline of the sterol ring system and the side chain attached extending from it in exactly the way predicted by Bernal. In the stable form A the two molecules overlap throughout their length and this obscures some details of the projection. The metastable variety B shows a more open

TABLE II
Parameters of the Iodine Atoms in Cholesteryl Iodide

A		B	
$x = .043$	$x' = -.043$	$x = .217$	$x' = -.217$
$y = .25$	$y' = .75$	$y = .25$	$y' = .75$
$z = .248$	$z' = -.248$	$z = .042$	$z' = -.042$

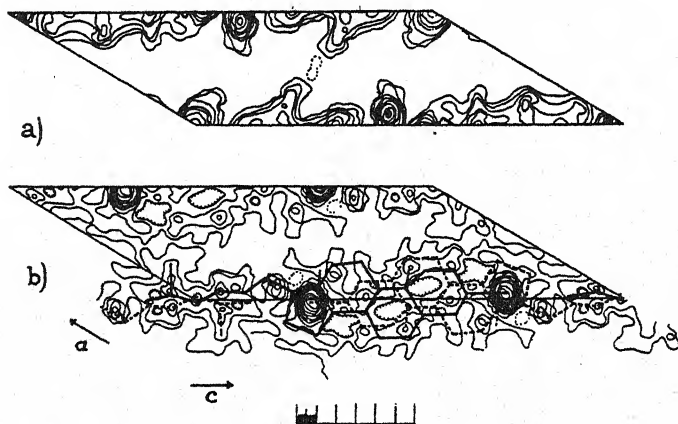


FIG. 1

Cholesteryl Iodide A

a) Patterson projection (P_{zz}) on (010)

The peak I. is that due to the iodine-iodine vector.

b) Projection of electron density (ρ_{zz}) on (010)

distribution of electron density and the position of almost every atom in the sterol skeleton is here fairly obvious. The relation between the two crystal structures is also clear (Fig. 3). In a single layer parallel to the plane of the projection the arrangement of the molecules is nearly the same, as suggested by the similarity in the dimensions a and c of the unit cell. The two structures differ principally in the relative arrangement of the two layers to one another in the unit cell. Structure A is derived from structure B by sliding one layer of molecules over the other a distance of 4.7 A.U. into rather closer packed positions. Since the projection of B shows a

clearer view of the sterol skeleton the X-ray analysis was continued on this form alone.

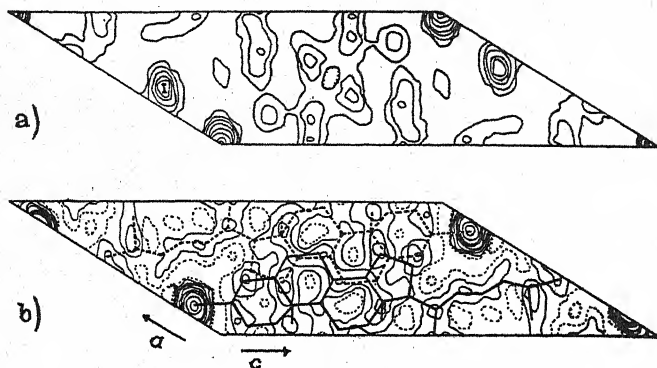


FIG. 2

Cholesteryl Iodide B

a) Patterson projection (P_{zz}) on (010)

The peak I. is that due to the iodine-iodine vector.

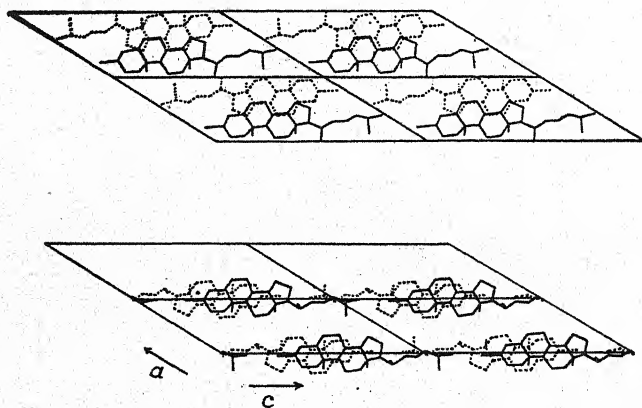
b) Projection of electron density (ρ_{xz}) on (010)

FIG. 3

Diagram to Show the Arrangement of the Molecules in Cholesteryl Iodide B (above)
and Cholesteryl Iodide A (below)
Atomic positions projected on (010)

From the Fourier projection two out of the three parameters of nearly every atom in the crystals can be approximately measured. The next step, the determination of the third parameter parallel to the b axis, is

much more difficult. The natural procedure is to calculate electron density curves parallel to the b axis running through the atomic positions found in the first projections. But here the difficulty mentioned above begins to operate. Parallel to the b axis the iodine atom positions are still related to one another by a centre of symmetry at the origin, though this is not true any longer for the positions of the molecules as a whole. The use of the phases of the iodine atoms alone introduces, therefore, a centre of symmetry into the structure. The consequence is that for every atom shown in these first three-dimensional electron density calculations a mirror image atom also appears, produced through the operation of the spurious centre of symmetry. The curves obtained, some of which are shown in Fig. 4, are symmetrical corresponding to possible carbon atom positions above or below the plane of the iodine atom at $y = \frac{1}{4}$. The direct calculations therefore only limit the positions of each atom in the unit cell to two possible alternatives.

Even so, and without pursuing the structure any further, it would be possible to make a few more deductions about the sterol skeleton. All the electron density curves show maxima not far from the plane $y = \frac{1}{4}$ corresponding to the presence of a roughly flat molecule. Some of the curves, *e.g.* $C\ 14$ and $C\ 15$, show maxima both in the region of $y = \frac{1}{4}$ and $y = \frac{3}{4}$. These maxima must represent atoms in different molecules since the distance between them is at least $\frac{1}{2}y$ or 4.5 A.U. The curves therefore distinguish atoms belonging to the two molecules in the unit cell from one another. Further, the distribution of the maxima about the plane $y = \frac{1}{4}$ is not regular; the molecule is clearly not exactly planar. And fourthly, there are signs of maxima in two regions some way from the plane $y = \frac{1}{4}$ near the suspected positions of the methyl groups of the sterol skeleton at $C\ 18$ and $C\ 19$.

For the final stages of the analysis some additional data had to be introduced, and here the natural assumptions that the normal distance between single bonded carbon atoms is 1.54 A.U., and the angle between their valencies the tetrahedral angle, were found sufficient. If the positions of two of the carbon atoms are fixed—in practice $C\ 10$ at $y = \frac{1}{4}$ and $C\ 1$ at $y = \frac{1}{4} + .09$ or 0.34 were chosen—it is possible to sort out the atoms belonging to one molecule from those of its mirror image with very little ambiguity. The result provides approximate positions for all the atoms in the molecule, from which a new set of now nearer true phase angles may be calculated. New calculations of electron density follow to fix the atomic parameters more exactly. Two types are shown. The first, the curves of electron density along lines parallel to b as before, are now no longer symmetrical (Fig. 4). The second, (Fig. 5), maps of electron density in three layers normal to the b axis, between them include all the atoms in the

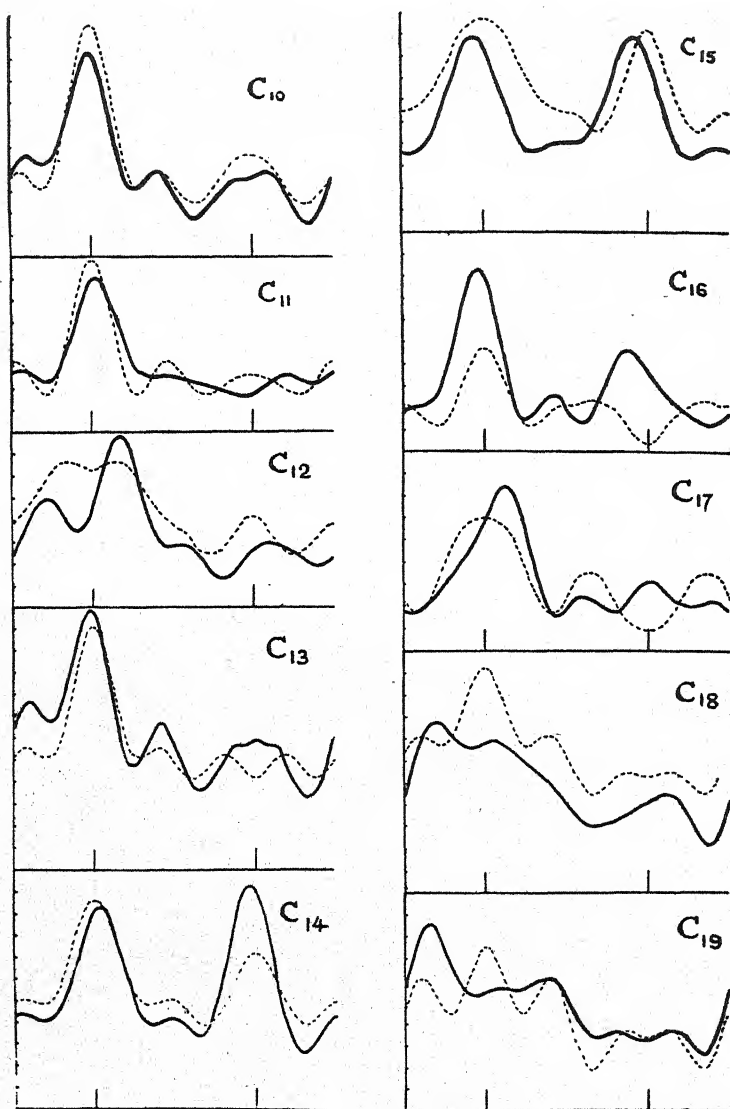


FIG. 4

Cholesteryl Iodide B

Curves showing electron density along lines parallel to *b* passing through atoms C 10 - C 19

The dotted lines are the curves calculated using the phases due to the iodine atoms alone. For the full curves the phases corrected for the carbon atom positions deduced were employed.

skeleton. Of these the map at the level $y = \frac{1}{4}$ is the most striking since it shows the five-membered character of ring D.

Putting together the evidence of the curves and maps we obtain the model of the sterol molecule photographed in Plate 1. The general form of the molecule is clear and is in good agreement with chemical views both in the linking together of the atoms in rings and in the non-planar char-

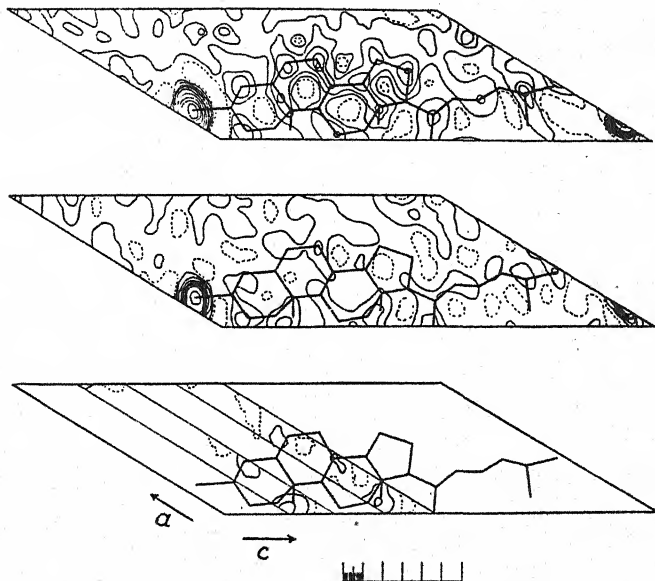


FIG. 5

Cholesteryl Iodide B

Sections showing electron density in planes parallel to (010) at heights $y = 0.25$ (a), 0.33 (b), and 0.12 (c). The section at height $y = 0.25$ shows most of the atomic positions since these are approximately in the same plane as the iodine atom. The remainder fall near the section at $y = 0.33$ with the exception of the methyl groups, near $y = 0.12$.

There are a number of spurious peaks in these sections due to the imperfections at the present stage of the analysis.

acter of these rings. The side chain continues the general line of the sterol ring system and is shown to be attached in the *cis* position to the methyl group at C 13, a point about which there has been some argument (65, 76). The stereochemical relation of the atoms C 13, C 16, C 17, C 20, C 21, and C 22 are now all established. As earlier suggested, in the one case through the preliminary crystallographic evidence (68), in the other by the work of Wieland and Dane (75 cf. also 29), the junction

between rings B and C is *trans* and also almost certainly that between C and D. The *trans* junction of C:D involves a slight but definite distortion of the valencies in the five-membered ring. A further distortion occurs over the ring system in the region of the double bond; it is noticeable that the atoms C 4, C 5, C 6, and C 10 all lie approximately in one plane. Here a note of caution must be inserted. During the course of the analysis it was assumed that the distance C 5:6 would be short, about 1.34 A.U., so that the double bond was to some extent "put in" to the molecule. But it is in fact difficult to correlate the possible atomic positions observed with any other double bond configuration. Finally the iodine atom is attached to C 3 and is in the "*trans*" position of Fig. 17 (*cis* to methyl at C 10).

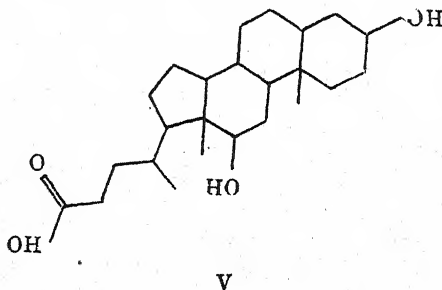
The crystallographic view of the structure founded on preliminary investigation is also largely confirmed. As suspected, the smallest refractive index α is nearly perpendicular to the plane of the ring system. In form A, γ also lies nearly along the molecular length but this is not the case in form B. The reason is clear since in form B, owing to the extended arrangement of the molecules in the *b* plane, the molecular length does not actually correspond to the direction of greatest atomic density in this plane.

One word remains to be said. This crystal structure determination, though it gives us the clearest view of the sterol skeleton so far obtained, is still a good way from being technically perfect. One reason for this is the instability of the crystals studied. The metastable form, particularly, gradually decomposes on keeping with loss of iodine, possibly due to the comparatively close approach of the two iodine atoms in the crystal. This has resulted both in a general weakening of the X-ray intensities and in experimental difficulties in measuring them exactly. While, therefore, considerable reliance can be put on the general conclusions of the analysis and particularly on the stereochemical deductions made, in detail refinement and confirmation would be very desirable.

III. THE INVESTIGATION OF THE CHOLEIC ACIDS

One group of steroids which differs stereochemically from the cholesterol type established by the analysis of cholesteryl iodide is constituted by the closely related group of the bile acids. Chemical evidence has proved that in the parent acid, cholic acid, the steroid ring system is completely reduced and the junction between ring A and B is almost certainly of the *cis* decalin type unlike the *trans* form found for the junctions B:C and C:D in cholesterol itself. While the investigation of this stereochemical difference is an important aspect of the crystallographic work in the bile acid series, perhaps the X-ray examination is even more interesting in the light that it throws on the problem of the constitution of the "choleic" acids.

In 1916, Wieland and Sorge (73) showed that the substance "choleic acid", which had been isolated from bile many years earlier, consisted of desoxycholic acid, V, and a small amount of a fatty acid, stearic or palmitic acid, in the proportion of 8 molecules of desoxycholic acid to 1 of fatty acid. Later work by Rheinbold (61, 62) and others, established the fact that certain bile acids and particularly desoxycholic acid could form a whole series of molecular compounds with various chemical substances amongst which were fatty acids and hydrocarbons. In the fatty acid series it was noticeable that the proportion of desoxycholic acid to fatty acid varied with the chain length of the fatty acid. The numbers of molecules of desoxycholic acid to fatty acids appeared to fall into well defined groups, 2, 3, 4, 6, and 8 which suggested an analogy with Werner's coordination compounds in inorganic chemistry.



In 1934, Go and Kratky examined by X-ray methods a whole series of choleic acids (38, 39), including the compounds of desoxycholic acid with stearic, palmitic, lauric, heptylic, butyric, and propionic acids. They found to their surprise that powder photographs of these compounds were practically indistinguishable. Accurate measurements of cell dimensions did show very small differences but these were only of the order of 1% as shown in Table III. Further, the intensities of the reflections given by all these compounds were nearly identical; accurate photometric measurements indicated small differences but these were hardly noticeable to the eye. It was clear that the crystal structure was based on the same essential molecular lattice throughout. The cell dimensions found corresponded to an orthorhombic crystal unit containing four molecules of desoxycholic acid¹ in all the compounds examined. But this cell was in the case of

¹ In dealing with this investigation I have renamed the crystallographic axes in order to point the relationship of this structure to the other steroid crystals mentioned. This is perhaps too bad as already two nomenclatures exist, that given by Steinmetz when the crystals were first isolated by Wieland and Sorge, and that used by Kratky and Go. The relations between a , b , and c as given here and a_s , b_s , c_s (Steinmetz) and a_k , b_k and c_k adopted by Kratky are as follows: $a = b_s = b_k$, $b = a_s = c_k$, $c = c_s = a_k$.

the longer fatty acids, stearic, palmitic, and lauric, too small to accommodate the complete fatty acid molecule. The relation is shown in Table III by the comparison between $M_{\max.}$, the unit cell molecular weight, and $M_{\min.}$, the unit cell molecular weight to be expected from the chemical analytical data.

These contradictory results have been explained in the following way. The crystal structure of the choleic acids is based on a framework of desoxycholic acid molecules through which run long canals containing the fatty acid molecules. These canals are parallel to the crystallographic b axis which is the direction of the greatest refractive index according to Steinmetz. Since this axis is short, only 7.2 A.U. long, the longer fatty

TABLE III
X-ray Measurements on the Choleic Acids

Fatty acid in choleic acid	a	b	c	v	ρ	$M_{\max.}$ (cryst.)	$M_{\min.}$ (chemical)	$\frac{M_{\max.}}{M_{\min.}}$	K
Stearic acid	13.53	7.23	25.90	2534	1.126	1729	3420	.506~ $\frac{1}{2}$	8
Palmitic acid	13.45	7.23	25.92	2521	1.122	1714	3392	.505~ $\frac{1}{2}$	8
Lauric acid	13.48	7.21	25.77	2505	1.126	1709	2552	.670~ $\frac{2}{3}$	6
Caprylic acid	13.49	7.21	25.75	2505	1.137	1726	1712	1.009~1	4
Heptic acid	13.52	7.22	25.77	2516	1.133	1727	1698	1.017~1	4
Butyric acid	13.49	7.23	25.80	2516	1.124	1714	1656	1.035~1	4
Propionic acid	13.57	7.23	25.79	2530	1.123	1722	or + 1 EtOH 1702	1.007~1	3
							1250	1.378~ $\frac{4}{3}$	
							or + 1 EtOH 1296	1.329~ $\frac{4}{3}$	

Here K = "coordination" number *i.e.* no. of molecules of desoxycholeic acid in the crystal per molecule of fatty acid.

acid chains must extend over several unit cells. It would be most natural to imagine the fatty acid chains filling these canals in a more or less irregular manner according to their chain lengths. This does not fit exactly with the idea of quite regular coordination numbers, though it would agree if the coordination numbers are in fact only approximately regular. Kratky and Go suggest that regularity of coordination numbers could be achieved if the fatty acid molecules tend to start only from a definite point in the crystal structure, leaving small gaps if the chain lengths did not quite fit the crystallographic interval available. Giacomello and Kratky (36) have further shown that correlation of the actual length of the b dimension, 7.2 A.U., with fatty acid chain length does give a quantitative explanation of the observed coordination numbers, except in the case of fatty acids above C_{21} . Here the X-ray data requires a higher coordination number

than 8, and it is possible that further chemical analysis might confirm this. Clearly, in any case, the analogy with Werner's coordination numbers in inorganic compounds is false. The numbers are a consequence of the crystallographic relationships.

There are problems for the crystallographer here. One might have expected that an increase in the size of the real crystal cell due to the presence of long fatty acid chains would appear on the X-ray photographs as very weak layer lines superimposed on the main structure. These

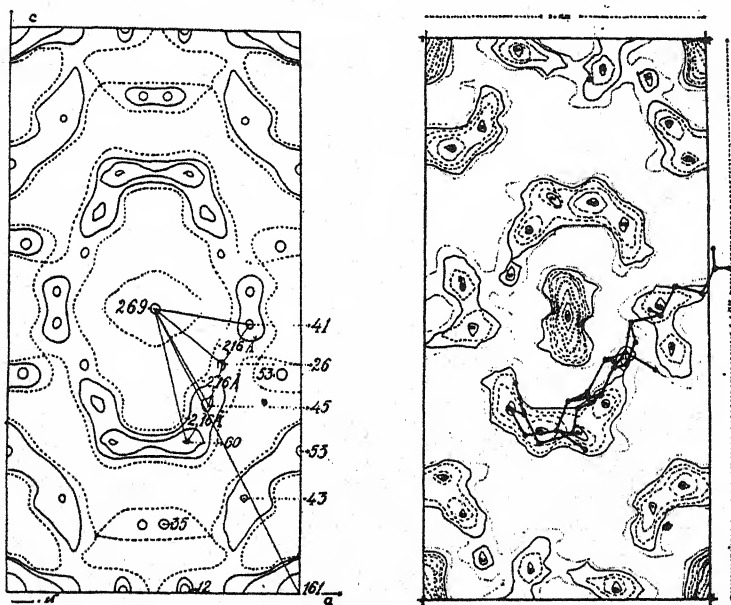


FIG. 6

Palmitic Acid-Desoxycholic Acid

- a) Patterson projection (P_{zz}) on (010)
 b) Projection of electron density (ρ_{xz}) on (010), with a Sketch of the approximate atomic positions projected on (010) superimposed

a) from *Rend. Accad. Lincei* 27, 101 (1938); b) mainly from *Gazz. chim. ital.* 60, 245 (1939).

have been sought for and not found; certainly their intensity would be very small, since the desoxycholic acid molecules which form the basic structure constitute 90% or more of its weight. On the other hand, there may actually be no superstructure if there is irregularity in the correlation of the sequences of the fatty acids in canals in different parts of the structure. The theoretical consequence of this, diffuse reflections along certain lines, would be even weaker and more difficult to detect than a superstructure.

The arrangement of desoxycholic acid molecules in the crystal unit which

makes this canal structure possible has been more fully investigated by Cagliotti and Giacomello (18). Giacomello estimated first the intensities of reflections of the type ($h0l$) and from the F^2 values derived a Patterson series as described above (37). This series shows a projection of peaks due to the main interatomic vectors in a plane normal to the b axis, the proposed direction of the fatty acid chains. Three points are noticeable in this projection, Fig. 6a. First, the main peak system consists of ridges 3.7 A.U. or more from the origin. Secondly, within the ridges the interval between peaks is about 2.16 A.U. Thirdly, there is a marked peak at the face-centred position. The effects agree well with the idea that there are fatty acid chains running normal to the plane of the projection and constituting, in projection, heavy scattering masses at the centre and corners of the unit cell. The appearance of the ridges can be explained if here the plane of the projection is also normal to the sterol ring system, the molecule in projection being therefore at least 3.7 A.U. either from the fatty acid

chains or from neighbouring desoxycholic acid molecules. Within each ridge it is noticeable that the distance 2.16 A.U. corresponds to the distance between groups of atoms around the ring junctions in the sterol-skeleton (Fig. 7).

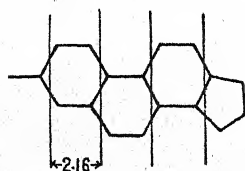


FIG. 7

Outline of Sterol Skeleton to Illustrate Prominent Interatomic Vectors Present

With the idea obtained of the molecular arrangement from this projection, Cagliotti and Giacomello constructed models and derived approximate values for the atomic parameters. From these parameters phase angles were calculated for the different X-ray reflections and a Fourier projection of electron density formed. In this part

of the X-ray analysis experimental errors can easily appear, and it is probable that in detail the molecular arrangement may be rather different from that employed by Cagliotti and Giacomello. The Fourier projection, shown in Fig. 6, is clearly extremely rough. It needs refinement, and this is very difficult to get purely by trial and error methods. Nevertheless, it does appear to give useful evidence on the choleic acid structure. It fits as expected with a view of the bile acid molecule projected parallel to the plane of the ring system. The four molecules in the cell are grouped in two pairs. In each pair the individual molecules form two sides of an arch so that they surround the fatty acid chain in the centre. The arch appears to be a consequence of the *cis*-decalin configuration of the ring junction A:B extended by the hydroxyl group at C 3 which is turned inwards towards the fatty acid chain. This hydroxyl group might accordingly supply the point of attraction to the chain postulated by Kratky and mentioned above. The second hydroxyl group at C 12 cannot be seen in the projection and is, presumably, in the plane.

of the ring system. There is evidence that the chain is attached to the ring system *cis* to the methyl group at C 13, a point which the later work on cholesteryl iodide has confirmed.

These choleic acid structures are clearly of an unusual and interesting type, and one which probably has a bearing on their physiological behavior. It is much to be hoped that the crystallographic study will be pursued further.

IV. THE CRYSTAL STRUCTURE OF CHOLESTERYL CHLORIDE AND BROMIDE

The X-ray analysis of cholesteryl chloride and bromide is in as great a state of incompleteness as that of palmitic-desoxycholeic acid. But it seems worth while giving some account of it, nevertheless, since the type of molecular arrangement found here is by far the most common in the sterol series.

Cholesteryl chloride and bromide both crystallize in thick monoclinic lath shaped plates elongated along the symmetry axis, b , the dominating face being the c face (001). Figure 8 shows the general appearance of the crystals as seen in the microscope and this appearance is common to a large number of sterol crystals. The crystal optics also are characteristic. The plane of the optic axes is (010) *i.e.* β is parallel to b while γ , the greatest refractive index, is inclined at a considerable angle to the main face. The unit cell dimensions measured are given in Table IVa. Here the direction of the c axis adopted from among those crystallographically possible is nearly along γ . Since in each unit cell there are only the two molecules required by the crystal symmetry (a two-fold screw axis) their orientation was established by Bernal following the optic orientation. The length of the molecules was placed nearly along $c(\gamma)$ and the plane of the sterol ring system parallel to $b(\beta)$.

This molecular arrangement received support from two other characteristics of the X-ray diffraction pattern. The first of these was quite unexpected. The X-ray photographs of both cholesteryl chloride and bromide show very marked diffuse reflections which appear as "smear" lines running through certain of the sharp X-ray reflections. These smear lines are particularly well marked on photographs of reflections of the type ($h0l$) where they are oriented along lines of constant l . It is clear that they correspond to diffuse reflections in certain planes, the normal to which in the crystal lies parallel to the c axis. The x and y coordinates of the

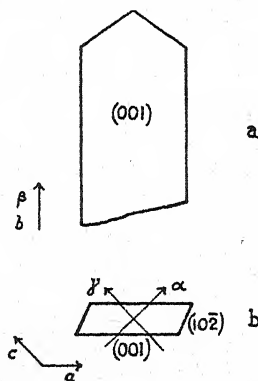


FIG. 8

Crystal of Cholesteryl
Bromide

- a) As usually seen in the microscope
- b) Cross section normal to b axis

molecules are fixed, but the z coordinate is to some degree variable. This variability can best be explained by the possibility of small molecular translations in the direction of the molecular length, and consequently provides confirmatory evidence that the length is nearly parallel to c . The disorder is not necessarily due to active movement of the molecules at normal temperatures, but may be frozen in as a result of the molecular arrangement present. It so happened that the phenomenon was first observed during a quite different investigation of substances showing liquid crystal formation, particularly of *p*-azoxy anisole and *p*-azoxy phenetole (8). It led the authors to look once again for the liquid crystal phases of cholesteryl chloride and bromide which are of the typically cholesteric variety, showing beautiful iridescent colours.

The other evidence on the molecular arrangement in these crystals comes from a study of the intensities of the X-ray reflections. The most striking feature of these is the strength of the reflection (200) and to a less degree (201). It is clear that a large proportion of the atoms must lie in these crystallographic planes. The effect is illustrated and amplified by the

TABLE IV_A
Unit Cell Dimensions of Cholesteryl Chloride and Bromide

	a	b	c	β	n	Space Group
Cholesteryl chloride	10.6	7.55	21.7	132°	2	P2 ₁
Cholesteryl bromide	11.0	7.55	21.6	134°	2	P2 ₁

Patterson projections calculated as before from the intensities of the ($h0l$) reflections which show the main vector pattern in the crystals projected on (010). In these diagrams (one is shown in Fig. 9a) there are two irregular series of maxima running along the lines $x = 0$ and $x = \frac{1}{2}$, and corresponding to two main sets of interatomic distances. One of these must be between atoms in the same molecule, which if they lie parallel to the c axis would have a common interatomic vector parallel to a of approximately zero. The other corresponds to distances between the two molecules where the common interatomic vector parallel to a is approximately $\frac{1}{2}$ (13).

Here we have clearly a projection derived from the same view of the molecules as was obtained by Cagliotti and Giacomello in the case of the choleic acids; only the molecule in this case is a sterol and not a bile acid. The plane of the projection is evidently normal to the sterol ring system and the molecules appear, as a consequence, essentially long and thin. Further, as in the choleic acid projection, the main ridge in the Patterson diagram, here at $x = 0$, is composed of a series of peaks 2.2, 4.4, 6.6 A.U. from the origin, and these distances correspond closely with the vectors expected between carbon atoms concentrated about the ring junctions in the sterol

ring system (Fig. 7). In this projection the peak heights are probably enhanced by the coincidence of the main carbon-carbon vectors with the carbon-halogen vectors.

The general orientation of the molecules in the crystal, and also roughly their dimensions, follow from these results. Crystallographically there remains to be fixed the z parameters of the molecules relative to one another. At this point the presence of the halogen atoms is of assistance. Comparison of the c plane intensities from cholesteryl chloride and bromide enables one to deduce that the halogen parameters must be small, of the order of .04 c . The peak x in the Patterson projection of cholesteryl bromide is therefore clearly due to the bromine-bromine vector and provides another measure of this quantity. The molecular arrangement which results is shown in Fig. 9b and also diagrammatically in Fig. 10. Here it is perhaps worth pointing out, in relation to this crystal structure, that the peaks due to the bromine-bromine and chlorine-chlorine vectors in the crystal are not immediately obvious in the Patterson projection as are the iodine-iodine vectors in cholesteryl iodide. While this is partly due to their absolutely smaller weight, it is also partly due to the overlapping of the carbon-carbon vectors in this particular view of the sterol skeleton. As a consequence it would be impossible to rely here on the phase angles calculated from the positions of the halogen atoms of one type alone to form electron density projections by Fourier series. A rough attempt has, however, been made at calculating such a series by a comparison of the intensities in the two structures, cholesteryl chloride and bromide (24 cf. 21). The result of this calculation, shown in Fig 9b, does confirm the general view of the sterol structure expected in the case of cholesteryl bromide. The configuration of the molecules appears to be the same as that found in cholesteryl iodide although it cannot be so accurately fixed here. In fact, in Fig. 9b it is a view of the cholesteryl iodide model which is inserted, with change of halogen atom, into the cholesteryl bromide unit cell. The halogen atoms are attached as before at C 3 and appear to have the same stereochemical configuration.

The crystal structures of cholesteryl chloride and bromide are closely related to those of cholesteryl iodide in the type of molecular packing present. The principal difference is a crystallographic one, the symmetry axis here being approximately parallel to, instead of approximately perpendicular to, the sterol ring system. The cholesteryl iodide variety is far more suitable for detailed X-ray analysis, since the molecular projection which is most easy to determine crystallographically, *i.e.* that on a plane normal to the symmetry axis, shows in outline the sterol ring system. But the fact that this symmetrical projection in the case of cholesteryl chloride and bromide shows the alternate view with the atoms concen-

trated along lines, had historical advantages. Here, unlike cholesteryl iodide, the direction of greatest atomic concentration was necessarily along

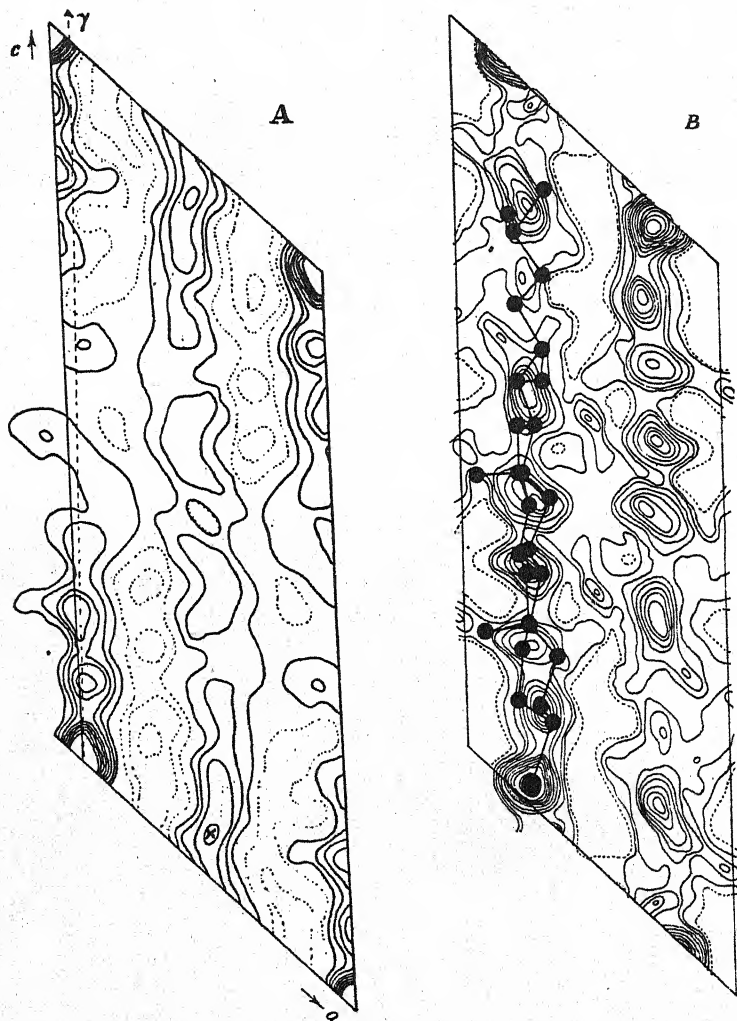


FIG. 9

Cholesteryl Bromide

A) Patterson projection (P_{zz}) on (010)

B) Very approximate projection of electron density on (010)

The view of the molecule superimposed on this projection is taken from the cholesteryl iodide model (Plate Ib). A slight rearrangement of the positions of the chain carbon atoms would give a better fit here.

From *Trans. Roy. Soc. (London)* **239**, 148 (1940).

the molecular length, and correspondingly the evidence provided by the optic orientation was a reliable guide to the molecular orientation. On this feature of sterol crystallography rested Bernal's deduction of the size of the sterol molecule.

V. A CLASSIFICATION OF STEROL CRYSTAL STRUCTURES

With the examination of cholesteryl chloride and bromide we return again to the early X-ray measurements on sterol crystals and the correlation of these with the relative molecular arrangement, size and shape. Both in these two crystal structures and in cholesteryl iodide the detailed analysis has served to confirm the general lath shaped form of the molecule. In both, the molecular dimensions can still be fairly described by the values of thickness, 4 A.U., width 7.5 A.U., and length 21 A.U., originally assigned to them;—values which again were little different from the corresponding figures deduced for the first sterols measured. In both, the molecular arrangements are a simple form of "lath close packing." But the two groups represent different crystallographic types of molecular arrangement

TABLE IVB
Unit Cell Dimensions of the Two Structure Types

	<i>a</i>	<i>a sin β</i>	<i>b</i>	<i>c</i>
Cholesteryl bromide	11.0	2×3.96	7.55	21.6
	<i>a</i>	<i>b</i>	<i>a sin β</i>	<i>c</i>
Cholesteryl iodide B	12.57	2×4.52	6.45	21.89

which it is useful to distinguish since the crystallographic units present here are fundamental to a large variety of sterol crystal structures.

The contrast between the two structures is clear from the cell dimensions repeated in Table IVb. As the detailed analysis has shown, the molecular dimensions can be fairly closely correlated with these cell dimensions. In cholesteryl bromide, approximately $a \sin \beta = 2 \times$ thickness, $b =$ width, $c =$ length. In cholesteryl iodide, $b = 2 \times$ thickness, $a \sin \beta =$ width, $c =$ length. The underlying difference between the two structures is more intricate, and probably depends on the deviation in the molecular character from that of "perfect laths." In the packing together of sterol molecules, the methyl groups projecting at right angles to the ring system must present something of a problem. In cholesteryl iodide B the staggering of the molecules in the b plane is very nicely arranged to avoid collision, but the packing is rather open and a closer adjustment is effected, maintaining the same molecular orientation, in cholesteryl iodide A. The difficulty of this orientation appears to be that the monoclinic symmetry axis is here nearly normal to the sterol ring system and necessarily brings two methyl groups between every pair of molecules parallel to this axis.

Closer fitting can be effected in the alternative structure type where the symmetry axis is parallel to the plane of the ring system and there are, between molecules, alternately four, and no methyl groups. This is perhaps one reason why this alternative structure type, that of cholesteryl chloride and bromide, is so much the most common in the main sterol series.

The distinction made between these two monoclinic structures, based on the relative orientation of the unique crystal symmetry axis to the molecular axes in the crystal, can be used as the basis of a general classification of sterol crystal structures. Naturally the classification cannot avoid being somewhat arbitrary since the molecules are not actually simple laths and the main molecular axes seldom if ever exactly coincide with the crystallographic axes. But certain relations between sterol crystal structures are so common that it seems worth while pointing them out.

To take first the cholesteryl bromide type of structure which is found so often that we have come to speak of it as the "normal" type (Fig. 10,—1). Here the monoclinic symmetry axis b corresponds in direction to the β refractive index, and the unit cell dimensions may be described, following the form above, as in general $a \sin \beta = n_t \times \text{thickness}$, $b = n_w \times \text{width}$, $c = n_l \times \text{length}$. For the classification the orientation in the crystal of the molecular axes, thickness, width and length (taken in order) to the crystal axes may be described by the symbols abc (also in order) followed by numerals $n_t n_w n_l$ to represent the multiplicities. For cholesteryl bromide the symbol is then $abc\ 211$ which may be conveniently shortened to $a\ 211$. The product of the figures gives of course the number of molecules in the unit cell.

Cholesteryl iodide, on the other hand, belongs to a much smaller group of crystals in which the monoclinic symmetry axis b is more nearly some multiple of 4.5 A.U., the thickness, and optically coincides with the direction of the least refractive index, α (Fig. 10,—2). This we call the reversed type with cell dimensions $b = n_t \times \text{thickness}$, $a \sin \beta = n_w \times \text{width}$, $c = n_l \times \text{length}$. The symbol for classification $bac\ n_t n_w n_l$ or more shortly $b\ n_t n_w n_l$ becomes for cholesteryl iodide $b\ 211$.

In neither cholesteryl bromide nor cholesteryl iodide does the plane of the ring system exactly coincide with the crystallographic planes suggested by the classification, and it is not surprising to find a number of sterol crystals which fall so clearly midway between the two types that it seems undesirably arbitrary to classify them as belonging either to one or to the other. These have been separated into a group to themselves, ab , or crossed. The length of the molecules is still along c but the plane of the ring system is probably nearly at 45° to b (Fig. 10,—3). The group symbol becomes $ab \cdot c(n_t \times n_w) \cdot n_l$. An example which is midway between cho-

lesteryl chloride and iodide is provided by Δ^4 -cholestene-7-ol (ψ -cholesterol) with cell dimensions $a \sin \beta = 2 \times 5.83$, $b = 6.15$, $c = 17.75$ A.U., structure type ab (21)1.

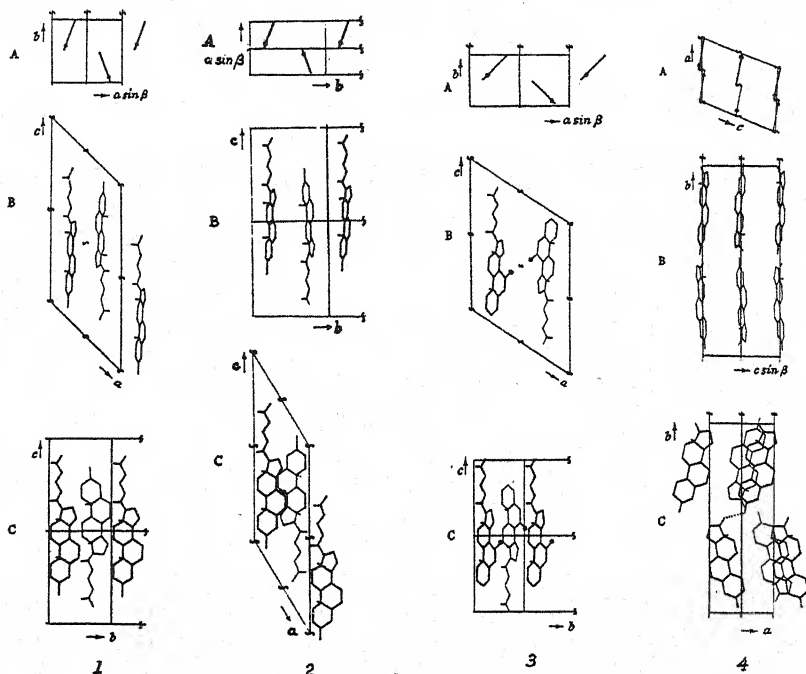


FIG. 10

DIAGRAM TO ILLUSTRATE STRUCTURE TYPES

1. Arrangement of the molecules in cholesteryl bromide, structure type a 211 (A) projection normal to (001), (B) projection on (010), (C) projection on (100).
2. Arrangement of the molecules in cholesteryl iodide B, structure type b 211. (A) projection on (001), (B) projection on (100), (C) projection on (010).
3. The probable arrangement of the molecules in Δ^4 cholestene-7-ol, structure type ab (21)1. (A) projection normal to (001), (B) projection on (010), (C) projection on (100).
4. The probable arrangement of the molecules in estrone 3, structure type c 212. (A) projection on (010). (B) projection normal to (100). (C) projection on (001). — molecule at 0, — molecule at $\frac{1}{2}$, - - - - - hydrogen or hydroxyl bond.

Partly from *Trans. Roy. Soc.* 239, 151 (1940).

Crystallographically a third type of structure is possible in which the monoclinic symmetry axis b is most nearly parallel to the molecular length. If the nomenclature of the crystal axis is arranged so that the directions of the molecular thickness, width, and length, are given by the axes c ,

a , and b respectively, the group symbol becomes cab , shortened to c , $n_1n_2n_3$. This is a very uncommon type of structure though a few examples are known (Fig. 10,—4).

This classification only applies strictly to monoclinic crystals containing lath shaped molecules. Actually about two-thirds of the steroid crystals examined so far are monoclinic so that it has a fairly wide validity; the remainder are triclinic and orthorhombic. It is in practice very easy to fit nearly all the triclinic structures into the same grouping since they usually show close relations to one or other of the monoclinic series.² The orthorhombic structures present more of a problem.

In the orthorhombic structures all three axes are symmetry axes, and so the structure might belong to any or all of the main divisions given above. An added complication is that here there is even less tendency for the direction of the molecular axes to coincide with the direction of the crystal axes. A case in point is provided by the bile acid framework in the structure of palmitic-desoxycholic acid. Here the general direction of the molecular length is at an angle of about 45° to the a and c axes. Nevertheless the crystals show certain relations to the normal type of sterol structure. The crystals are platy, elongated along an axis (b) 7.2 A.U. long which is shown by the X-ray analysis to correspond mainly with the direction of the width of the bile acid ring system. There are deviations naturally from the normal sterol type, due to the presence of the *cis*-decalin ring system; and the usual optic orientation is, of course, destroyed by the presence of the fatty acid. But the relation can still be traced here, and it is even more marked in certain other sterol structures. These have, therefore, been grouped into the main divisions established above, following generally secondary considerations such as crystal habit.

This form of classification was suggested first as a result of the general survey of steroid structures carried out by Bernal, Crowfoot, and Fankuchen. In the original paper (13) 105 sterol crystal structures are listed and related to one another. Table V is a form of abstract of this collection. It gives the main varieties of crystal structure which have been found with one compound to illustrate each type. Inside each main division a , b , ab and c the subdivisions are based first on the varying multiplicities of n , and secondly on the space groups shown. The symbols fall into a reasonable sequence into which new structures can be inserted as found without disturbing the general order. Table V does not, however, give a good perspective view of steroid crystallography. In the original table the relative importance of the a or normal structures was much more obvious. Of 105 compounds listed, 78 fell into this division. And among

² An exception is cholesteryl salicylate, but here the molecule may deviate considerably from the typical lath shape (43).

TABLE V
A Classification of Steroid Crystal Structures

Structure Type	Space Group	n	Compound	Thickness	Width	Length			
				$a \sin \beta$	b	c	β	a	$c \sin \beta$
<i>abc normal:</i>									
a 112	P2 ₁	2	β -Ergostadienetriol I	5.85	7.16	2×16.5	119°	6.74	28.77
a 211	P1	2	β -Cholestene dibromide	2×4.18	7.7	20.4	127 $\frac{1}{2}$ °	10.5	18.2
	P2 ₁	2	Cholesteryl bromide	2×3.96	7.55	21.6	134°	11.0	15.5
a 212	P1	4	Cholestanol 2H ₂ O	2×4.7	7.76	2×18.4	106°	9.79	35.42
	P2 ₁	4	Ergosterol H ₂ O	2×4.40	7.57	2×19.25	115°	9.92	34.82
	C2	4	α -Spinasterol acetate	2×4.71	7.69	2×20.1	119°	10.75	35.2
	A2	4	Ergosterol acetate maleic anhydride adduct I	2×4.85	9.5	2×16.6	112°	10.5	31.0
	P22 ₁ 2 ₁	4	Coprostone	2×5.42	7.62	2×15.15	90°	10.85	30.3
	P2 ₁ 2 ₁ 2 ₁	4	Dibromocholesteryl chloride	2×4.03	7.7	2×21.4	90°	8.07	42.8
a 214	A2	8	Ostreasterol	2×4.5	7.65	4×19.65	117°	10.10	72.0
	P2 ₁ 2 ₁ 2 ₁	8	β -Spinasterol H ₂ O	2×5.15	7.98	4×18.6	90°	10.30	74.3
a 218	A2	16	Cereviserol I	2×4.9	7.6	8×18.5	93°	9.8	148.0
a 411	P2 ₁	4	Pyrocalciferol ?	4×4.55	7.15	20.5	92°	18.20	20.4
	P2 ₁ 2 ₁ 2 ₁	4	Cholesterylene	4×3.97	7.66	19.25	90°	15.85	19.25
a 412	P2 ₁	8	Calciferol	4×5.02	7.2	2×17.8	102°	20.5	34.8
	C2	8	Calciferol-pyrocalciferol	4×4.97	7.35	2×17.7	100°	20.2	34.9
a 414	A2	16	γ -Sitosterol H ₂ O	4×4.5	7.53	4×20.6	120°	20.44	71.4
	P2 ₁ 2 ₁ 2 ₁	16	α -Cholestanetriol II	4×6.51	7.43	4×17.75	90°	26.05	71.0
a 812	P2 ₁	16	β -Dihydrofucosterol	8×4.8	7.62	2×18.0	94°	38.5	35.9
<i>bac reversed:</i>									
b 211	P2 ₁	2	Cholesteryl iodide B	2×4.52	6.45	21.89	149°	12.57	11.3
b 221	P2 ₁	4	Cholesteryl acetate	2×4.7	2×8.1	17.6	103°	16.6	17.1
	P2 ₁ 2 ₁ 2 ₁	4	Cholestene hydrochloride	2×4.45	2×7.3	19.2	90°	14.6	19.2
b 222	P1	8	Anhydrous cholesterol	2×5.23	2×6.2	2×18.9	117 $\frac{1}{2}$ °	14.0	33.5
	P2 ₁ 2 ₁	8	Suprasterol II	2×5.20	2×6.7	2×17.7	90°	13.4	35.4
<i>(ab)c crossed:</i>									
ab (21)1	P2 ₁	2	Δ^4 -Cholestene-7-ol	2×5.83	6.15	17.75	123 $\frac{1}{2}$ °	13.96	14.80
ab (21)2	P1	4	Cholesterol MeOH	2×5.1	6.23	2×21.15	124°	12.3	34.9
	P2 ₁	4	α -Ergosterol	2×6.05	6.1	2×17.8	93°	12.1	35.6
	A2	4	Dicholesteryl ether	2×5.32	6.23	2×38.15	110°	11.3	71.3
ab (22)1	P2 ₁	4	Cholestane	10.85	11.0	19.8	104°	11.2	19.25
	P2 ₁ 2 ₁ 2 ₁	4	Dibromo-cholesteryl bromide	12.0	12.30	18.25	90°	12.0	18.25
ab (41)2	C2	8	cis- $\Delta^{4,6}$ -Cholestene-3,4-diol	4×6.65	5.85	2×15.3	120°	30.8	26.5
ab (8)2	C2	16	Cholestane-6-ol-EtOH	14.8	19.7	2×16.35	106°	15.4	31.4
ab (8)4	C1	32	Cholesterol H ₂ O	14.6	19.1	4×18.5	112 $\frac{1}{2}$ °	15.8	68.4
ab (82)2	C2	32	Anhydrous ergosterol	4×12.9	12.56	2×16.4	101°	52.7	32.1
<i>cab:</i>									
c 112	P2 ₁	2	Hydroxyketone from cholesterol (androstane-3(β)-ol-17-one)	6.3	6.62	2×11.05	109°	—	—
c 212	P2 ₁	4	Estrone 3	2×4.61	7.60	2×11.05	112°	—	—
c 221	P2 ₁	4	Cholestene	2×5.5	2×6.6	19.45	94°	—	—

the *a* group two structure types occurred most frequently and have a special importance, *a* 211 and *a* 212. The relation between these two is interesting.

The structure type *a* 211, 18 compounds, (the cholesteryl bromide type) is one of the most homogeneous groups listed and consists almost entirely of ether, ester, halogen and keto derivatives. The structure type *a* 212 (36 compounds) can be considered as built up from two units of the *a* 211 type placed end to end in the *c* direction. This can be achieved in two ways to give orthorhombic and monoclinic symmetry respectively, and the significance of the two is rather different. In the main monoclinic series, seventeen compounds belonging to the space group $P2_1$ (and for convenience $P1$), the doubling of *c* doubles the asymmetric unit and suggests that here the molecules are united end to end by hydroxyl bonds. Fig. 18 shows the characteristic double layer structure which results, drawn for the type structure, ergosterol- H_2O . In the orthorhombic group, on the other hand, the asymmetric unit is the molecule itself and, while it is possible for end to end association to occur in the space group $P22_12_1$, this is not the case with $P2_12_12_1$. The group of compounds here is similar to those found in *a* 211 while the monoclinic double layer series consists almost entirely of steroids having terminal hydroxyl groups. Similar relations between double and single layer structures can be paralleled in other parts of the table; compare, for example, Δ^4 -cholestene-7-ol, *ab* 211, with α -ergosterol, *ab* 212. The crystallographic classification points here to a chemical difference which we shall find recurring as we deal from a chemical point of view with these main groups of steroids.

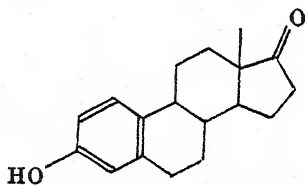
VI. THE SEX HORMONES

The X-ray investigation of the sex hormones began in 1932 almost simultaneously with that of the sterols proper. At that time even the molecular formulae of these compounds were unknown, but their relation to the sterols, which was proposed shortly afterwards, was supported by the X-ray evidence available (5). The first compounds to be examined, estrone, VI, and estriol, VII, were followed by equiline (34), androsterone, testosterone, and progesterone (12) as each hormone in turn was isolated. But the chemical investigation rapidly outstripped the scope of the early X-ray work, and the crystallographic study has been left on one side in the years between.

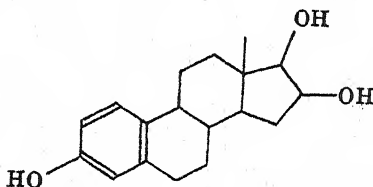
The compounds examined in this group are only 12 in number, and it therefore seems worth while to give the crystallographic data on them in full in Table VI. This includes the six hormones mentioned above together with six compounds which are closely related to or derived from them. The most immediately striking point about this table is the variety

of crystal structures represented. Only two, estrone 3, and estriol are actually isomorphous. The variety is presumably associated with the relative shortness of the molecules compared with those in the main sterol series—the lath character is less pronounced, and chemical differences may be more effective. Even quite different types of molecular arrangement may have much the same stability. In spite of this it is fairly easy in all cases to deduce the approximate orientation of the molecules to the crystal axes, and this is shown by the classification symbol in the last column of Table VI. Between them these compounds show all the main divisions of sterol crystal structure.

The variation in crystal structure is particularly well illustrated by the structures of estrone itself. Here three polymorphic modifications have been distinguished which show three quite different types of molecular arrangement. The relation between them is illustrated diagrammatically in Fig. 11. The first modification, the orthorhombic stable form estrone 1



VI



VII

is only grown from the vapour phase, and the small rectangular plates which form are often markedly elongated along the *c* axis unlike almost all other steroid crystals examined (47, 48). The arrangement of the molecules must approximate closely to that shown in Fig. 12. The three refractive indices have been measured and show greater differences between them than do those of the other two modifications of estrone, as shown in Table VII (47, 48, 54, 55, 70). This is an indication that here the molecular axes are nearly parallel to the crystal axes. The strong intensity of the reflection (400) confirms this view. So far only one other steroid examined, cholesterylene, shows a parallel structure. The other two estrone modifications are more normal. In the second orthorhombic form, estrone 2, the birefringence is much lower and clearly there is very considerable slanting of the molecules with reference to the crystal axes. The third modification, estrone 3, which is monoclinic was the one first to be measured crystallographically by Bernal (5). The crystal plates are similar in appearance to many sterol crystals but the symmetry axis *b*

TABLE VI
Sex Hormones and Related Substances

Substance	Space group	#	a	b	c	β	Optic sign	Optic orientation	Structure Type
Equiline		4	9.06	6.42	23.5	90°	—	$a = \alpha, b = \beta, c = \gamma$	a212
Estrone 1	P2 ₁ 2 ₁ 2 ₁	4	16.28	7.46	12.15	90°	—	$a = \alpha, b = \beta, c = \gamma$	a411
Estrone 2	P2 ₁ 2 ₁ 2 ₁	4	9.9	7.7	18.2	90°	—	$a = \alpha, b = \gamma, c = \beta$	a212
Estrone 3	P2 ₁	4	7.60	22.1	9.22	112°	—	$b = \gamma, \alpha$ 10° from \perp (001)	c212
Estriol	P2 ₁	4	7.50	22.8	9.06	112°	—	$b = \gamma, \alpha$ 17° from \perp (001)	c212
Bromo-methoxyestrone	P2 ₁ 2 ₁ 2 ₁	4	9.0	13.8	12.8	90°	—	$a = \alpha, b = \beta, c = \gamma$	b221
Androstane-3(β)-ol-17-one	P2 ₁	2	6.62	22.1	6.3	109°	—	$b = \gamma, \alpha$ 14° to c in acute <	c112
Androsterone	P2 ₁	2	9.45	7.7	11.95	111°	+	$b = \beta, \gamma$ c 8° from \perp (001) towards c	a211
Testosterone	P2 ₁	4	14.73	11.09	11.01	125°	+	$b = \beta, \gamma$ nearly along c	ab (22)1
α -Progesterone	P22 ₁ 2 ₁	4	10.27	13.88	12.31	90°	+	$a = \alpha, b = \beta, c = \gamma$	b221
Pregnandiol	P2 ₁ 2 ₁ 2 ₁ ?	4	10.2	7.3	24.6	90°	+	$a = \alpha, b = \beta, c = \gamma$	a212
Pregnane	P2 ₁	4	12.0	6.29	22.6	100°	+	$b = \beta, \gamma$ nearly \perp (001)	ab (21)2
3-Chlor-androstanone I mpt. 175°	P2 ₁ 2 ₁ 2 ₁	4	10.73	11.41	13.80	90°	+	$a = \alpha, b = \beta, c = \gamma$	(ab) 221
3-Chlor-androstanone II mpt. 128°	P2 ₁ 2 ₁ 2 ₁	4	12.41	10.67	12.56	90°	+	$a = \alpha, b = \beta, c = \gamma$	

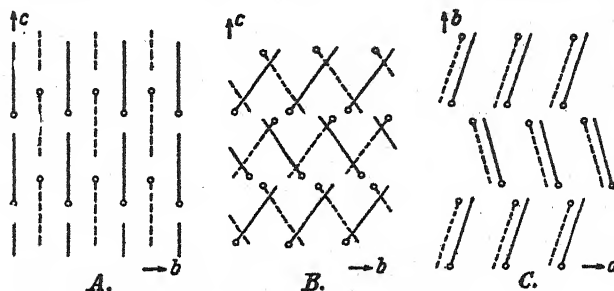


FIG. 11

Interrelationship of the Three Polymorphic Forms of Estrone
The molecules lie approximately flat in the plane of the paper.

Molecule at 0 —————

Molecule at $\frac{1}{2}$ ————

(Except in the case of estrone 1 where only molecules at $\frac{1}{2}$ and $\frac{3}{4}$ respectively are shown.)

From *Z. Krist. A*, 93, 471 (1936).

is normal to the main face and follows the length of the molecule. The type of arrangement present here has already been illustrated in Fig. 10,—4.

In spite of their differences, these structures, and indeed all the sex hormone structures, have one feature in common: they are all characteristically single layer structures. In none is there evidence of association between chemically identical groups tending to double the apparent molecular length.³ The implication of this became clear as evidence accumulated that the hydroxyl group of estrone was in the same position as the hydroxyl group of cholesterol, namely C 3. In the sterol structures, wherever there is a hydroxyl group at C 3 it is usual to find that the crystal structure is a double layer structure caused by association between the terminal hydroxyl groups. The fact that such association does not occur in the sex hormone crystal structures led Bernal to suggest that there must be active groups placed at opposite ends of the molecule (5). In all the crystal structures of estrone it is possible then, as the figures show, for hydrogen bonds to exist between the ketone and hydroxyl groups in neighboring molecules. This

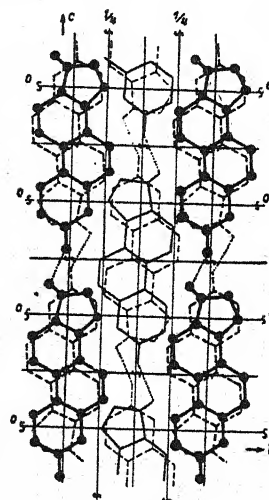


FIG. 12

Suggested Structure of
Estrone 1, Projected on
(100)

Molecules at $\frac{1}{4}$ —•—•—

$\frac{3}{4}$ ————

$\frac{5}{4}$ ————

$\frac{7}{4}$ - - - - -

Hydrogen bond.....

From *Z. Krist. A* 93, 467 (1936).

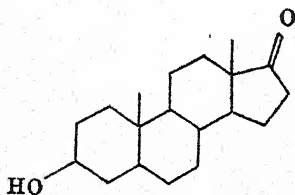
³ With the possible but not probable exception of equiline on which the published data is incomplete.

feature of sex hormone structure has since received ample chemical proof.

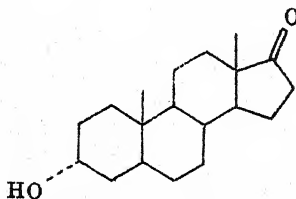
Chemically the most striking evidence of the relation of the sex hormones to the sterols was provided by the degradation of epicholesterol to androsterone by Ruzicka and his co-workers in 1934 (64, cf. also 17, 32). The identity of the androsterone, VIII, obtained in this preparation with the naturally occurring hormone was checked by X-ray measurements, and it is perhaps a nice point that androsterone itself crystallizes in a single layer structure of the normal sterol type, a 211. The isomeric compound, androstane-3(β)-ol-17-one, IX, obtained from cholesterol, on the other hand, shows an unusual and interesting structure type. The crystals are elongated along the b axis but this direction of elongation as in estrone

TABLE VII
Refractive Indices of Estrone and Estriol

Substance	α	β	γ
Estrone 1	1.511	1.621	1.697
Estrone 2	1.594	1.628	1.647
Estrone 3	1.520	1.642	1.690
Estriol	1.533	1.642	1.686



VIII



IX

1 proves to be that of the molecular length. There are only two molecules in the unit cell, and these are placed vertically above one another along the b axis. The orientation of the molecules perpendicular to this axis is fixed by the optic orientation and the very strong reflection (10 $\bar{2}$). Chemically androsterone and androstane-3(β)-ol-17-one differ only in the stereochemical configuration of the hydroxyl group. There is, in both crystal structures, some indication of this configuration, but it is far from conclusive. In androsterone the molecular thickness derived in the usual way is rather large and the molecular length rather short, effects which might be explained if the hydroxyl group projects from the plane of the ring system. In androstane-3(β)-ol-17-one the unusual crystal habit suggests relatively easy growth of the crystals parallel to b . This growth

might be favored by the hydrogen bond system, particularly if the hydroxyl group followed the plane of the ring system; compare for example estrone 1. But while these conclusions agree with present chemical views, they are little more than speculations, and require exact X-ray analysis for their confirmation or rejection. The crystallographic relations in androstane-3(β)-ol-17-one are so simple that it might be worth while here to attempt a straightforward trial and error analysis. The corresponding chlorides (19) unluckily crystallize in orthorhombic structures which are more difficult to deal with (Table VI).

VII. THE HEART POISONS

The heart poisons of the digitalis group were first examined crystallographically in 1934 (9). Strophanthidin and the toad poisons, bufagin and cinobufagin, followed soon after in 1935-6 (22, 23). The main purpose of the measurements was to see if some relation could be traced through the crystallographic data between these compounds and the sterols and sex hormones and, as in the case of the sex hormones, the research has not been pursued since. Only a limited number of compounds were measured, and all of these are listed in Table VIII.

The first obvious feature of the cardiac aglucones seemed to be their dissimilarity from most of the sterols in crystallography, particularly in optic character in relation to unit cell dimensions. This was not surprising in view of the possible functional groups present, particularly tertiary hydroxyl groups which might reasonably be expected to destroy most of the correlation established in the main sterol series. A derivative was therefore examined in which these groups were removed. It was a lactone prepared by dehydration of digoxigenin and reduction of the product, and had the chemical composition $C_{23}H_{36}O_2$ (X). It proved to crystallize in the α 211 structure type and it was correspondingly easy to deduce approximate dimensions for this molecule as in the case of similar sterol crystals. These dimensions fitted well with the idea that the genins contained the cyclopenteno-phenanthrene skeleton with a lactone ring attached to it at C 17. Fig. 13 shows a drawing of the proposed crystal structure. The dimensions found entirely excluded any molecular structure in which the lactone ring was attached to ring B of the sterol skeleton as had been earlier suggested in the case of strophanthidin (30).

Now that the chemical structures of the digitalis group of genins are reasonably well established it is possible to review the remaining crystallographic data in a different way. The proposed formulae (33) for the four genins, dianhydro-gitoxigenin, XI, digitoxigenin, XII, digoxigenin, XIII, and gitoxigenin, XIV, are given below. Of these the crystal structure of the first, dianhydro-gitoxigenin also appears to be of the α 211 type. In

TABLE VIII
Heart Poisons

Substance	Space group	<i>n</i>	<i>a</i>	<i>b</i>	<i>c</i>	β	Optic sign	Optic orientation	ρ	M
Lactone from digoxigenin (octahydro-trianhydro-digoxigenin), X	P ₂ ₁	2	10.6	7.7	11.7	101°	+	$b = \beta, \gamma 23^\circ$ from \perp (001)		
Dianhydro-gtioxigenin, XI	P ₂ ₁	2	9.62	7.85	12.8	93½°	-	$b = \gamma$		
Digitoxigenin, XII	P ₂ ₁	4	18.13	7.16	14.95	90°	-	$a = \beta, b = \alpha, c = \gamma$		
Digitoxigenin, XIII	P ₂ ₁	4	9.62	16.75	12.85	90°	+	$a = \beta, b = \alpha, c = \gamma$		
Gitoxigenin, XIV	C ₂	8	6.16	13.3	53.3	98°	+	$\perp c = \beta, b = \alpha, c = \gamma$		
Strophanthidin A	P ₂ ₁	12	19.54	7.41	43.9	90°	+	$b = \alpha$		
Strophanthidin B + ½H ₂ O	C I	4	13.70	18.22	8.91	71½°	+	$b = \alpha, \gamma 8^\circ$ from c	1.240	446±9
Bufagin + EtOH	P ₂ ₁	2	13.54	7.29	15.97	131°	-	$a = \alpha, b = \gamma, c = \beta$	1.261	447±10
Cinobufagin	P ₂ ₁	4	7.61	15.79	19.45	90°		$a = \gamma, b = \beta, c = \alpha$	1.229	487±10
Acetylcinobufagin	C ₂ ₂ ₂ ₁	16	28.74	8.14	44.76	90°		$a = \beta, b = \gamma, c = \alpha$	1.280	443±10
Cinobufagone	P ₂ ₁	8	21.79	8.62	24.22	90°				

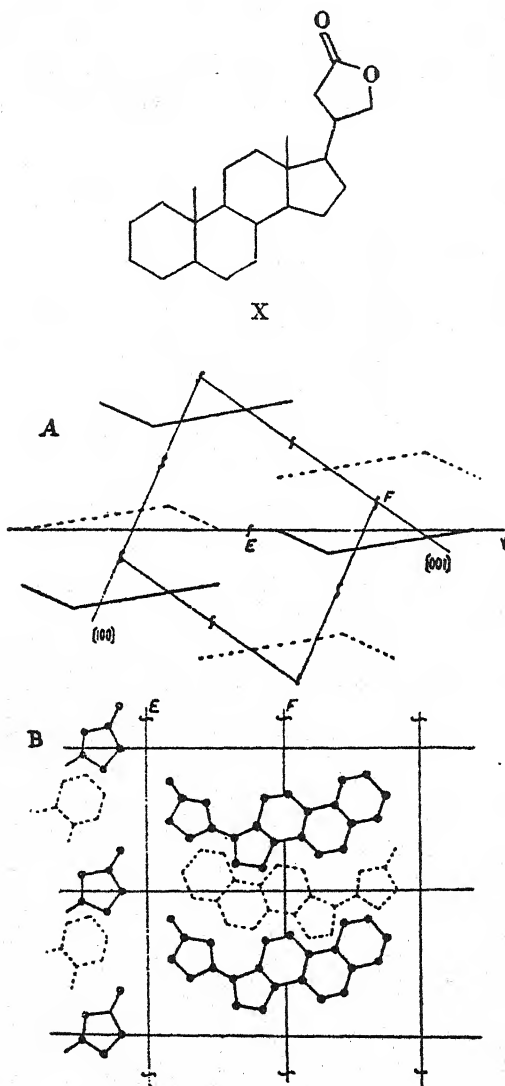


FIG. 13

Probable Molecular Arrangement in Lactone from Digoxigenin

A: Projection on (010).

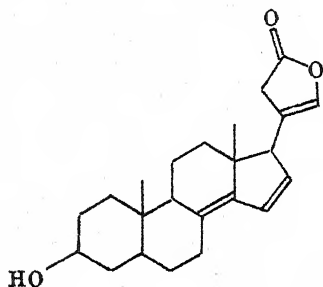
B: Diagram to show arrangement along [010]

(Projection approximately between [010] and γ direction)

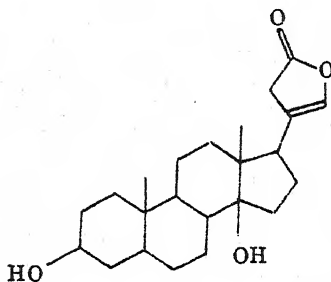
1. —•—•— Molecule in plane of paper.
2. —○—○— Molecule slightly behind 1, related to it by screw axis E.
3. — — — Molecules halfway in front of or behind 1

From *Chemistry and Industry* 53, 953 (1934).

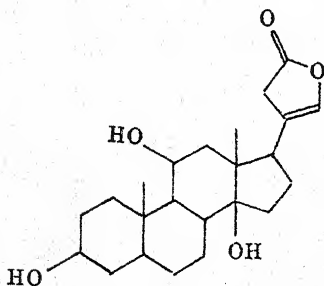
habit the crystals are usually not of the normal variety; needles bounded by the faces $(10\bar{2})$ and $(20\bar{1})$ are the most common form. The optics also deviate but this deviation can probably be correlated with greater slope than usual of the molecule in the bc plane. The most important feature, however, is that as in the sex hormones the crystal structure is a single layer one, which suggests that the hydroxyl group which has been shown to be present at C 3 interacts in the crystal with the lactone carbonyl



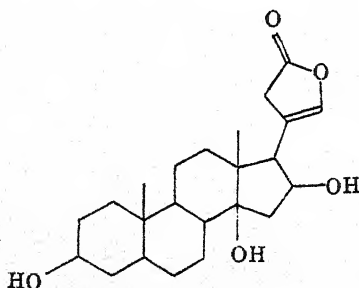
XI



XII



XIII



XIV

group of the succeeding molecule. The same is true of both digitoxigenin and digoxigenin. These crystal structures are orthorhombic, and it is much less easy to predict the molecular arrangement. The crystal optics deviate considerably from the normal, which can probably largely be accounted for by the tertiary hydroxyl groups present, combined with considerable staggering of the molecule to the crystallographic axes. Digoxigenin certainly also contains solvent of crystallization. In the

fourth compound, gitoxigenin, the crystal structure is quite different, a double layer structure. The molecular dimensions are closely controlled by the cell dimensions and space group found, and these provided strong evidence, even in the original investigation, both of the position of the lactone ring in this genin and of the existence of one hydroxyl group at C 3. The molecular arrangement suggested is shown in Fig. 14. In the original drawing the position of the second and third hydroxyl groups were not, of course, inserted. It seems reasonable to suggest now that one of these, the hydroxyl group at C 16 can interact with the lactone carbonyl group in the neighboring molecule in the crystal. The interaction requires only slight rearrangement of the molecular positions from those first given. This would account for the fact that only in this structure is the hydroxyl group at C 3 free to form a double layer.

The crystallography of strophanthidin, XV, is very difficult to correlate with its molecular structure, beyond saying that both are more complicated than in any other steroid examined. Two forms have been observed. Strophanthidin A from ethyl acetate has a very complex orthorhombic arrangement, strophanthidin B, from methyl alcohol and water, is triclinic (31) and contains $\frac{1}{2}$ H₂O per molecule of strophanthidin (45).

The toad poisons, bufagin and cinobufagin, were first measured in order to determine their molecular weights. The empirical formulae for the two compounds were originally given as C₂₈H₃₆O₆ and C₂₉H₃₈O₇, but in 1932-3 Jensen and his collaborators put forward instead the formulae C₂₄H₃₂O₅ and C₂₅H₃₂O₆ as more probable (46, 20). The X-ray measurements on bufagin confirmed Jensen's view (cf. also 26). The crystallographic unit had a molecular weight of 446 ± 9 agreeing with the value 446 calculated for the formula C₂₄H₃₂O₅ (400) + one molecule of alcohol of crystallization (46) which was proved to be present. In the case of cinobufagin the molecular weight found of 447 ± 10 (22) suggested that the formula might be C₂₆H₃₄O₆ (442) and showed it was certainly not C₂₅H₃₂O₆ (428). This was confirmed by the subsequent examination of acetyl cinobufagin and cinobufagin (23) (see Table VIII).

All three cinobufagin crystal structures are very complex and cannot give any direct information from the preliminary data on the molecular struc-

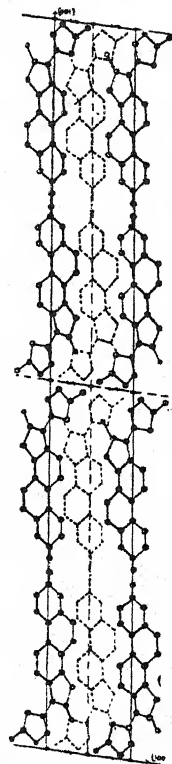
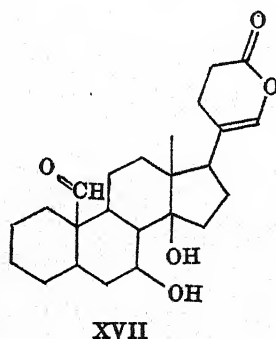
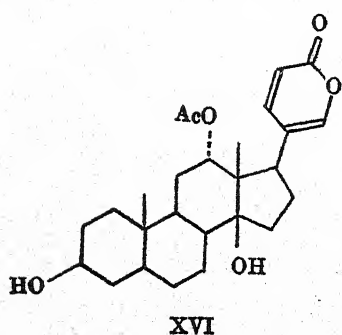
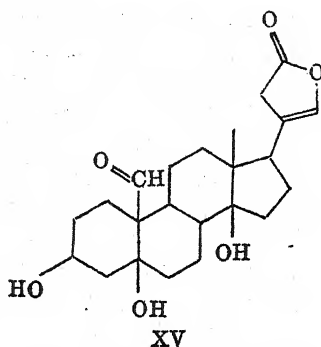


Fig. 14

Diagram of Structure Proposed for Gitoxigenin (XIV) Projected on (010)

From *Chemistry and Industry* 53, 953 (1934).

ture, for which formula XVI has been proposed (49, 71). But bufagin again appears to have the α 211 type of crystal structure. In habit the crystals are of the normal variety, and it seems reasonable to suppose that the fact that here α , the smallest refractive index, is parallel to b , is due to the presence of *e.g.* a tertiary hydroxyl group and not to any considerable deviation in molecular arrangement. The molecular dimensions were



therefore given as $4\frac{1}{2} \times 7 \times 16$ A.U., with a query on the position of the alcohol of crystallization in the unit cell. From these dimensions one would have expected a rather longer molecule than that represented by the formula XVII proposed by Fieser (33). But the presence of the alcohol of crystallization makes it impossible to be certain at this stage what is the actual length of the molecule itself. Again it should be noticed that the crystal structure is of a single layer type.

VIII. THE STEROLS

Of all the steroids examined by the X-ray methods by far the greatest number belong to the main sterol series. Nearly 100 different sterols and sterol derivatives have been studied, and since the information on the major part of these has been collected together by Bernal and others (13) no attempt will be made to reproduce the accumulated data here.

Chemically the compounds measured present a very assorted collection. They have been gathered from almost all the main laboratories working on sterol structure, and many obvious gaps exist in the chemical sequence. For example, of the stereoisomers of cholestanol, three only have been measured, cholestanol itself, epicholestane and coprosterol, but not the fourth, epicoprosterol. Measurements on epicholesterol and allocholesterol are also obviously badly needed for comparisons between the cholesterol series and other groups such as that of the photo-derivatives of ergosterol.

Crystallographically too these compounds show wide variations in structure types. Some of them, *e.g.* the crystal structures of cholesterol and of coprosterol, are very complicated and could hardly be expected to yield much exact chemical information. Others, particularly the halogen derivatives of cholesterol, are comparatively simple and have already been analyzed in some detail. Certain of the crystal structures found are very individual, and here the compounds present could easily be recognized by simple morphological examination of their crystals. Morphological measurements were, for example, made by Steinmetz to confirm the identity of the hydrocarbon obtained synthetically by Wieland and Jacobi from cholanolic acid with coprostane prepared by Windaus from cholesterol. Hydrated cholesterol itself and cholestene hydrochloride form even more easily recognizable crystals. But the greater number of sterol crystals have such close similarity that optical and morphological measurements are of little use in distinguishing them one from another. In all, however, it has been proved possible by the X-ray measurements to fit molecules corresponding in size and shape to those represented by the present chemical formula, the reduced cyclopenteno-phenanthrene ring system with side chain and substituents of varying complexity.

Among the compounds studied, the halogen derivatives of cholesterol form the group in which the most obviously beautiful crystals are to be found. These crystals were many of them isolated in the course of Mauthner's researches on the reactions of the double bond in cholesterol and of the hydroxyl group. Morphological descriptions of several of them occur in the course of the original papers. One good example, cholestene hydrochloride drawn by Becke and Karny from the crystals prepared by Mauthner (52, 1) is reproduced in Fig. 15. Another is α -cholestene dibromide (51). The chief interest in the X-ray investigation of these compounds is

naturally in attacking the same two points in chemical structure, the position of the double bond and of the hydroxyl group in cholesterol.

Probably the best crystallographic evidence on the position of the double bond in cholesterol is that obtained from cholesteryl iodide. But additional evidence is supplied by the examination of cholesteryl chloride hydrochloride, XVIII. The crystal structure of this is extremely similar to that of cholesteryl chloride or bromide, but the intensities of the X-ray reflections differ considerably. The change can be followed by forming as before a Patterson projection on the b plane. Compared with the corresponding projection of cholesteryl bromide (Fig. 9), the new pro-

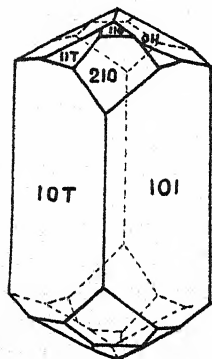


FIG. 15

Crystal of Cholestene
Hydrochloride

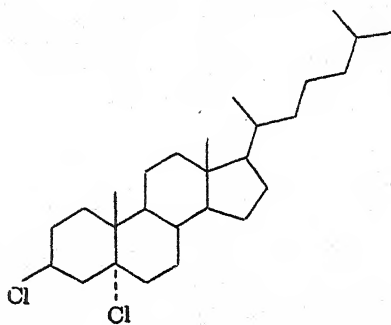
Here the nomenclature of the crystal axes has been changed from that used by Becke and Karny to conform with the general scheme.

From *Süßber. Acad. Wien* 116, IIb, 1019 (1907).

jection (Fig. 16) shows an additional line of peaks due both to new chlorine-chlorine interatomic distances and also to new carbon-chlorine distances. Fig. 16 illustrates an arrangement of the molecules that would account for the observed effects. From a chemical point of view the most significant feature is that the new line of peaks is midway between the original lines, suggesting that the extra chlorine atom projects at right angles to the skeleton; and peak A, from its strength, probably corresponds to the chlorine-chlorine distance within the molecule. This distance is 4.2 A.U. and fixes the position of the chlorine atom and therefore one end of the double bond system in the neighborhood of C 5, as would be expected from chemical theory.

In discussing the position of the hydroxyl group there are two points to be considered, first its point of attachment to the sterol ring system, and secondly its stereochemical configuration. Starting with a reduced skeleton and a *trans*-configuration of the junction between rings A and B, two possible configurations for a hydroxyl group attached at C 3 have been distinguished. In one, which following Ruzicka, Furter, and Goldberg (66), we may call *cis*, the hydroxyl group projects from the plane of the ring system, in the other, *trans*, it should lie almost in the plane of the ring system (Fig. 17). The same distinction may be continued when there is a double bond impinging on the ring junction as in cholesterol itself.

Unfortunately, the evidence that the iodine atom in cholesteryl iodide is attached to C 3 and has the *trans* configuration does not establish corresponding facts about the hydroxyl group in cholesterol. In the first place the iodide used was actually made in an indirect way by a rather



XVIII

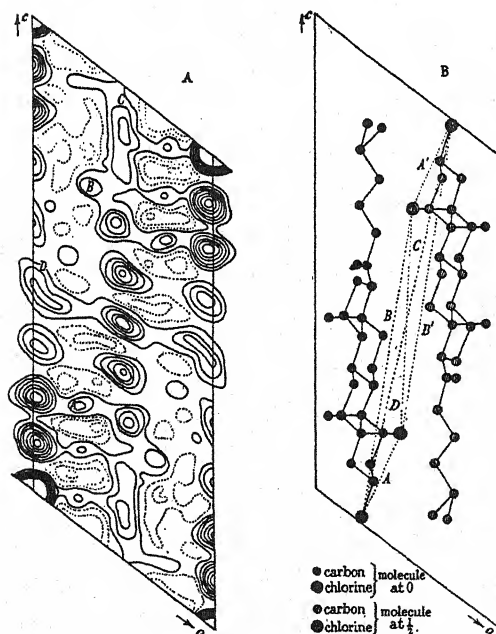


FIG. 16

Cholesteryl Chloride Hydrochloride

(A) Patterson projection, P_{zz} . (B) Probable arrangement of the molecules, projected on (010).

The dotted lines show chlorine to chlorine distances.

From *Trans. Roy. Soc. (London)* 239, 173 (1940).

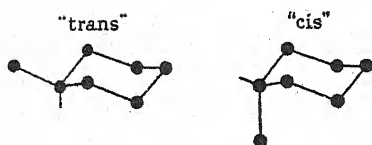


Fig. 17

The Stereochemistry of the Sterol Hydroxyl Group at C 3 curious reaction from *i*-cholesterol (14). As a similar reaction yields cholesteryl chloride and bromide and as an apparently identical iodide has since also been prepared from cholesterol (42), we may reasonably take it that the hydroxyl group in cholesterol is attached to the same carbon atom as the halogen atom, *i.e.* C 3, but there are additional difficulties in accepting its configuration. Reduction of cholesteryl chloride gives a compound, α -chlorcholestane, which is identical with the product obtained by the action of phosphorus pentachloride on epicholesterol, while a different compound, β -chlorcholestane is obtained through the action of this reagent on cholestanol (67). Clearly Walden inversion must have occurred at least once, and the configuration of the hydroxyl group in cholesterol cannot be correlated for certain with the position of the halogen atom in the cholesteryl halides, although there are indications, both chemical (3) and crystallographic in favor of their identity in configuration. Only one point can be made. From its crystal unit cell dimensions and even more from the intensities of the *c* plane reflections it is clear that it is α -chlorcholestane that has the same configuration as cholesteryl chloride and that no inversion occurs during the reduction.

Historically the first crystallographic evidence that the hydroxyl group in cholesterol was at C 3 came from the fact that in the crystal the molecules have a double layer arrangement. The crystal structures of all the forms of cholesterol are rather complicated, and a simpler example to take is ergosterol-H₂O shown in Fig. 18. Still better evidence is supplied by the direct comparison of the crystal structure of dicholesteryl ether with, for example, those of ostreasterol and actiniasterol. These all crystallize in

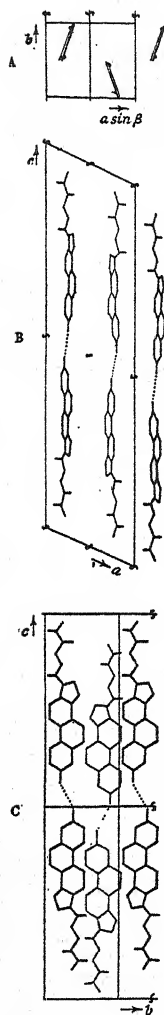


Fig. 18

The Probable Arrangement of the Molecules in Ergosterol H₂O Structure Type a 212

(A) Projection normal to [001].

(B) Projection on (010).

(C) Projection on (100).

From *Trans. Roy. Soc. (London)* 239, 156 (1940).

the same space group, A 2, but whereas in dicholesteryl ether the molecule is the asymmetric unit, in the two sterols the unit is a double molecule. In all three structures the length of the asymmetric unit is approximately $\frac{1}{2}c$ and is clearly double the normal length of a sterol molecule (cf. table IV, A and B). In dicholesteryl ether the doubling of the molecular length is produced by the chemical elimination of water between the two hydroxyl groups, and these must be terminally placed to account for the effect observed. In ostreasterol and actiniasterol the same applies, only the doubling is due here to hydroxyl bonds between the two corresponding hydroxyl groups. Actually the appearance of a double layer crystal structure is not conclusive evidence that there is a terminal hydroxyl group present—or even a hydroxyl group at all. Occasionally compounds such as esters or even hydrocarbons (*e.g.* pregnane and ergosteryl acetate (69)) show, presumably for reasons of convenience of packing, apparently double layer structures. On the other hand it is very rare to find a hydroxyl group at C 3 and no double layer unless there is some other active group capable of hydrogen bond formation in a different direction, as in the sex hormones. The only exception so far appears to be pyrocalciferol, and even

TABLE IX

	<i>a</i>	<i>b</i>	<i>c</i>	β	<i>n</i>
Dicholesteryl ether	11.30	6.23	76.3	110°	4
Ostreasterol	10.10	7.65	78.6	117°	8
Actiniasterol	10.12	7.58	82.0	121°	8

here the asymmetric unit is doubled in another direction—and also here the data are very poor.

In the formation of double layers the terminal hydroxyl groups may interact either directly with one another or through water molecules, as in the case *e.g.* of the oxalic acid crystal structures where both types of interaction are known (44, 58). Among the sterols there appears to be difficulty about the first process. It is not easy to obtain most sterol crystals anhydrous, and when these are prepared they seem to show very complex crystal structures (*e.g.* anhydrous cholesterol and ergosterol). All the normal sterol crystal structures contain solvent of crystallization, usually water, sometimes methyl alcohol which probably plays a similar part. The case of ethyl alcohol of crystallization is more doubtful. It does certainly interact with the sterol hydroxyl groups in some structures, notably *i*-cholesterol + EtOH which is exceedingly similar to *i*-cholesterol ethyl ether, both in cell dimensions and in the intensities of the X-ray reflections (2). But here the crystal structure is of an essentially single layer type. The nearest comparable structure in the sterol series is that of dibromocholesteryl chloride (Table X).

One might expect that crystal structure would be one of the properties of a molecule most sensitive to stereochemical changes, such as that in the configuration of the hydroxyl group at C 3 passing from the *cis* to the *trans* series. There is in fact every indication that this is so; cholestanol and epicholestanol are crystallographically quite distinct and parallel differences appear between other compounds belonging to the two series. Unfortunately it is impossible from the preliminary X-ray examination to be quite certain which group has the *cis*, which the *trans* configuration. This is another point on which more detailed X-ray analysis is needed.

The group of compounds examined which show the most marked similarities to one another in crystal structure is, as would be expected, the group of naturally occurring sterols in all of which there is a hydroxyl group at C 3, usually in the same configuration. These sterols differ from one another in such features as the number of double bonds present and the number of carbon atoms in the side chain. The difference in molecular weight of sterols belonging to the different series can usually be detected through accurate measurement of the crystal unit cell dimensions

TABLE X

	<i>a</i>	<i>b</i>	<i>c</i>
<i>i</i> -cholesterol + EtOH	8.20	7.6	42.4
<i>i</i> -cholesterol ethyl ether	8.23	7.58	43.3
Dibromocholesteryl chloride	8.07	7.7	42.8

In all cases $n = 4$, Space Group $P2_12_12_1$.

and densities. Actually the sterol crystals themselves are not very suitable for these molecular weight determinations for the reason discussed above. They contain solvent of crystallization, the weight of which ought to be deducted from the crystal molecular weight, but which is difficult to determine accurately owing to the frequently very hygroscopic character of the anhydrous crystals. Better measurements can be carried out on derivatives such as hydrocarbons, ketones, and acetates. Thus the carbon content in the ergosterol series can be established from the measurements on ergotetraene; that in the cholesterol series from cholestone, and so on. Some of the measurements carried out are listed in Table XI.

Both the differences and the similarities between the different members of these sterol series are shown by the X-ray crystallographic data. The fundamental crystallographic unit throughout is the double layer structure of the type $a 212$ shown in the case of ergosterol-H₂O in Fig. 18. The cell dimensions of the sterols listed in Table XIIa are either roughly equal to or some multiple of the ergosterol-H₂O cell dimensions, and even where these multiplicities occur they appear more as a superstructure on

TABLE XI
Molecular Weight Determinations in the Sterol Series

Compound	a	b	c sin β	ρ	No. in unit cell	No. in asymmetric unit	Molecular wt. measured	Molecular wt. calculated	Formula
Cholestenone	α' 14.63 \pm 0.05	7.86 \pm 0.015	d_{001} 10.26 \pm 0.02	1.078 \pm 0.002	2	1	385 \pm 3	384	C ₂₇ H ₄₄ O
Ergotetraene	7.73 \pm 0.04	9.67 \pm 0.04	16.00 \pm 0.05	1.065 \pm 0.005	2	1	384 \pm 7	378	C ₂₈ H ₄₂ O
γ -Spinasterol acetate	13.28 \pm 0.02	6.95 \pm 0.03	31.0 \pm 0.20	1.064 \pm 0.004	4	1	460 \pm 8	456	C ₃₁ H ₅₂ O ₂
Ergosterol H ₂ O	9.92 \pm 0.04	7.57 \pm 0.03	34.82 \pm 0.04	1.055 \pm 0.003	4	2	18+401 \pm 7	396	C ₂₈ H ₄₄ O
Stigmasterol H ₂ O	9.51 \pm 0.09	7.59 \pm 0.02	36.64 \pm 0.06	1.055 \pm 0.004	4	2	18+406 \pm 7	412	C ₂₉ H ₄₈ O
γ -Sitosterol H ₂ O	20.44 \pm 0.10	7.53 \pm 0.02	71.40 \pm 0.20	1.035 \pm 0.002	16	4	18+415 \pm 6	414	C ₂₉ H ₄₈ O
β -Sitosterol H ₂ O	10.33 \pm 0.10	7.55 \pm 0.01	35.00 \pm 0.10	1.049 \pm 0.004	4	2	18+414 \pm 7	414	C ₂₉ H ₄₈ O
β -Sitosterol acetate	10.43 \pm 0.04	7.63 \pm 0.02	36.4 \pm 0.1	1.049 \pm 0.002	4	2	460 \pm 6	456	C ₂₉ H ₄₈ O \cdot CO \cdot CH ₃

TABI
 Higher Plant an

Compound	Space Group	<i>n</i>	<i>a</i>	<i>b</i>	<i>c</i>	
Ergosterol H ₂ O	P2 ₁	4	9.92	7.57	38.5	
Zymosterol H ₂ O	P1	4	9.65	7.57	35.51	β
γ -Sitostanol (and $\alpha\beta$ -sitostanol?) 2 H ₂ O	P1	4	9.94	7.74	37.4	γ
γ -Sitosterol H ₂ O	A2	16	20.44	7.58	82.5	
β -Sitosterol H ₂ O (from rubber)	P2 ₁	4	10.33	7.55	41.3	
Stigmasterol H ₂ O	P2 ₁	4	9.51	7.59	37.2	
Actiniasterol VI (lugworm sterol)	A2	8	10.12	7.58	82.0	
Cervisterol I	A2	16	9.8	7.6	148.0	
Cervisterol II	P2 ₁	4	10.6	7.45	39.0	
Ostreasterol	A2	8	10.1	7.65	78.6	
Brassicasterol (impure preparation)	—	—	9.6	7.7	37(d ₀₀₁)	
β -Dihydro-fucosterol	P2 ₁	16	38.5	7.62	36.0	
Spinastanol 2H ₂ O (?)	P1	4	10.00	7.74	37.5	α
α -Spinastanol 2H ₂ O (?)	P1	4	10.4	7.35	37.4	
β -Spinastanol H ₂ O	P2 ₁ 2 ₁	8	10.30	7.28	74.3	

 TABI
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Compound	Space Group	<i>n</i>	<i>a</i>	<i>b</i>	<i>c</i>	β	
Calciferol	P2 ₁	8	20.5	7.2	35.6	102°	1
Pyrocalciferol-calciferol	C2	8	20.2	7.35	35.4	100°	1
Dihydrocalciferol	P2 ₁	4	11.5	7.26	36.4	117°	1
Lumisterol	P2 ₁	4	20.3	7.25	38.4	152½°	1
Suprasterol I	C2	8	25.0	7.5	34.8	129°	1
Suprasterol II	P2 ₁ 2 ₁	8	13.4	10.4	35.4	90°	1

E XIIa

of Animal Sterols

	Morphology	Optics	Structure Type
115°	Laths, {010}, {001}, {102}, {104}	$+$, $b = \beta$, γ 62° from (001)	$a212$
106°	Plates, {001}	$+$, b near β , γ 59° from (001)	$a212$
108°	Plates, {010}, {001}, {101}, {111}	$+$, $b = \beta$, γ 81° from (001)	$a212$
120°	Laths, {010}, {001}, {100}, {120}	$+$, $b = \beta$, γ 65° from (001), 2E large	$a414$
122°	Rhomb-shaped plates, {001}, {111}	$+$, $b = \beta$, γ inclined to (001)	$a212$
100°	Rhomb-shaped plates, {001}, {111}, {201}	$+$, $b = \beta$, γ 78° from (001)	$a212$
121°	Plates, {001}, {111}, or {101}, {111}	$+$, $b = \beta$, γ inclined to (001)	$a214$
103°	Laths, {010}, {001}, {101}, {111}	$+$, $b = \beta$, γ nearly \perp to (001)	$a218$
120°	Laths, {010}, {001}, {101}, {111}	$+$, $b = \beta$, γ 58° from (001)	$a212$
117°	Irregular plates, {001}	$b = \beta$, γ inclined to (001)	$a214$
	Very thin irregular plates giving very few X-ray reflexions	$b = \beta$, γ inclined to (001)	$a212?$
94°	Laths, {010}, {001}, {101}, {411}	$b = \beta$, γ c. 80° from (001)	$a812$
90°	Laths, {010}, {001}, twins, $\rho = 1.031$	β 7° from [010], γ inclined to (001)	$a212$
106°	Plates, {001}, {211}, {101}, {011}	$+$, $b = \beta$, γ nearly normal to (001), $2V = 70^\circ$	$a212$
103°	$\rho = 1.032$		
100°	Prisms, {010}, {001}, {101}, {111}	$-$, $a = \alpha$, $b = \beta$, $c = \gamma$, $2V = 70^\circ$	$a214$
	$\rho = 1.030$		

E XIIb

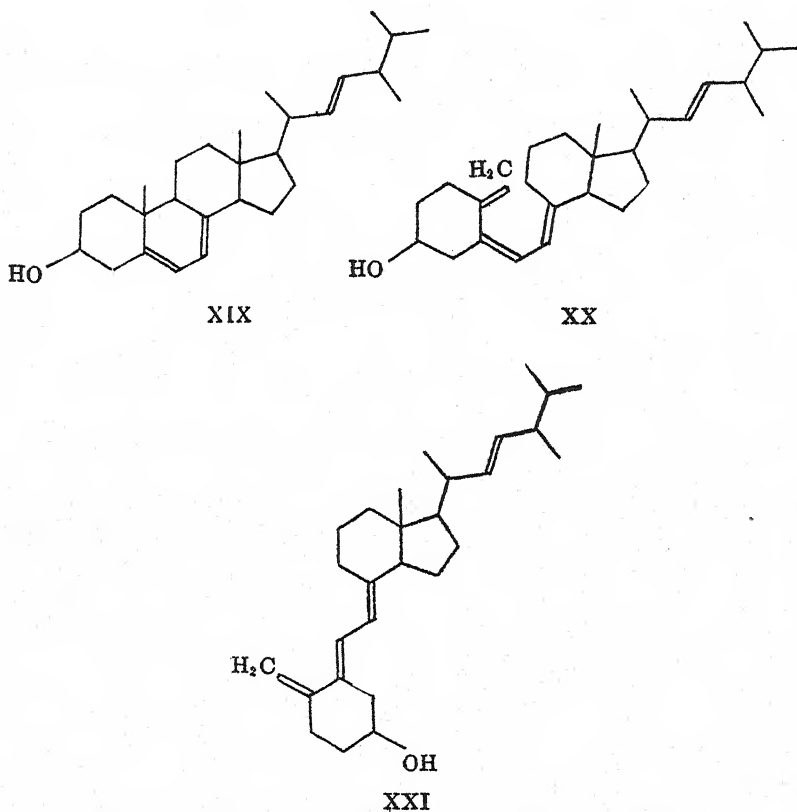
of Ergosterol

Morphology	Optics	Structure Type
Laths, {010}, {001}, {011}, {101}	$+$, $b = \beta$, γ nearly \perp to (001)	$a412$
Laths, {010}, {001}	$+$, $b = \beta$, γ nearly \perp to (001)	$a412$
Laths, {010}, {001}	$+$, $b = \beta$, γ 64-60° from (001)	$a212$
Needles, {010}, {001}, {101}, {102}	$+$?, $b = \beta$, γ 31° from (001), 2E small	$a212$
Laths, {010}, {001}	? normal	$a412$
Prisms, {010}, {001}	$+$, $a = \alpha$, $b = \beta$, $c = \gamma$, 2E small	$b222$

the main unit which is indicated throughout by the stronger X-ray reflections. Plate II shows some X-ray photographs of ergosterol-H₂O which illustrates the characteristics of the diffraction effects obtained from all the compounds in this group. The general similarities in the intensities of the stronger X-ray reflections indicate that the same general molecular skeleton and arrangement are present throughout. But the intensities of the weaker X-ray reflections particularly are very sensitive to small changes in the crystal structure, and consequently the X-ray photographs of different individuals, while generally similar, are usually quite distinct. The measurements of β -sitosterol illustrate the point. The β -sitosterol examined had been isolated originally from rubber and was thought at first to be a terpene alcohol related to amyirin. The form of the X-ray reflections observed justified its classification as a sterol closely related to ergosterol long before its identity with β -sitosterol was established. Between other members of the group greater differences exist. Compare for example the photographs in Plate III of γ -sitosterol and stigmasterol with the corresponding photographs of ergosterol. There is a marked change in the position of the strong reflections in stigmasterol which corresponds to change in the slope of the molecules to the *c* plane. Stigmasterol is correspondingly easier to separate from sterol mixtures.

The X-ray crystallographic investigation of the sterols has covered in the last 10 years a good deal more ground than could have been envisaged when the first measurements were begun. From the initial discovery that sterol structure was not what it was thought to be the method has naturally been turned to more detailed examination of the problem both through the survey of sterol crystals in general and through the detailed analysis of particular structures. But at the end we return to the point from which the research set out. What is the position of calciferol in the sterol series? Table XIIb gives the unit cell dimensions of calciferol, calciferol-pyrocalfiferol, and dihydrocalfiferol. All of these show differences from the main ergosterol group, but hardly greater differences than might be expected from the change of configuration of the hydroxyl group. The most obvious alteration is in the relative increase in the molecular thickness, as deduced from the optic orientation and cell dimensions, together with an apparent decrease in molecular length (10). This did in fact affect Bernal's original calculation of 'sterol' dimensions. Compare for example the figures $5 \times 7.2 \times 17\text{--}20$ A.U. with those derived from cholesteryl chloride and bromide $4 \times 7.5 \times 21$ A.U. But in spite of the difference, there is still a very general similarity in certain of the X-ray reflections, particularly those from the *c* plane in calciferol and ergosterol (Plate IV). It is difficult to escape from the conclusion that the

relative positions of the atoms in the molecule are largely unchanged in passing from ergosterol, XIX, to calciferol. This can only be reconciled with the present formulation of calciferol (50, 53, 79) if the molecule retains in the crystal a configuration similar to that shown in the formula XX and not XXI. While at first sight this seems physically unlikely there are other indications in its favor. Crystallographically the matter can



only be finally settled by the detailed analysis of a derivative of calciferol or of one of the other vitamin D compounds. This could easily be carried out following the scheme adopted for cholesteryl iodide if only a suitable heavy atom derivative could be obtained. It seems worth suggesting that if possible this derivative should be one which leaves the configuration of the hydroxyl group unchanged. A single analysis might thus also settle one of the other outstanding problems of sterol structure.

IX. CONCLUSION

The sterols are so widely distributed in nature and many of them of such importance in biological processes that any method capable of throwing more light on their structure might be welcomed on their account alone. But the use of X-ray methods here described has also a wider interest as an illustration of the application of a new tool in chemical research. It is clear that the structure of the sterol skeleton was a particularly suitable subject for crystallographic study. The rigidity of the framework, the possibility of making a variety of derivatives with very little alteration in the fundamental molecular structure, the characteristic molecular shape, all are favorable circumstances. Obviously in not all fields of organic chemistry could such significant and far reaching conclusions have been drawn from relatively so little experimental evidence as were derived from the first X-ray study of the sterols. But the later methods of analysis developed during the course of the research, and particularly the use of heavy atoms as in cholesteryl iodide appear to be capable both of wide application, and of giving more detailed information.

From the analysis of cholesteryl iodide it is already possible to say something of the general scope of this method of attacking problems of molecular structure. It is, of course, limited by the crystallographic condition that the contribution of the heavy atom to the intensity of any particular X-ray reflection should outweigh that of all the other atoms in the structure. This does not mean that the heavy atoms must actually outweigh all these other atoms, since it is seldom in an organic compound that a maximum scattering effect is obtained. But it does mean that there is an upper limit to the size of the molecular structure which can be directly analyzed by this method. Clearly no atom on this earth is heavy enough to determine by its contribution alone the phase angles for reflections from protein crystals. But there are still a large number of problems which might be solved in this way on the structure of molecules of the same order of complexity as the sterols.

There is another limitation of a rather different kind on the degree of accuracy which can be achieved in X-ray analysis of these complex molecular structures. The knowledge of the relative atomic positions which we obtain at the end of the analysis of cholesteryl iodide may fairly be described as organic chemical rather than physicochemical, if the distinction may be made. It is possible to find which atom is attached to which in the structure and roughly their orientation in space, but not to measure exact interatomic distances or bond angles. One reason for this is the order of complication of the molecular structure. The positions of the heavy atoms only approximately determine the phases in three dimensions,

and the complete solution still depends on the exact measurement of too many parameters to be achieved at present. But this difficulty of making exact measurements may also depend partly on the character of the crystal structures themselves. It seems probable that in a complex crystal structure of this kind the atomic positions are actually not so precisely ordered throughout space as they are in simpler examples. One indication of this is the relative weakness of reflections from planes of small spacing in cholesteryl iodide; another is the existence of smear lines among the reflections from cholesteryl chloride and bromide. It is presumably the weakness of the intermolecular forces combined with the size of the molecules that is responsible for this degree of disorder within the crystals.

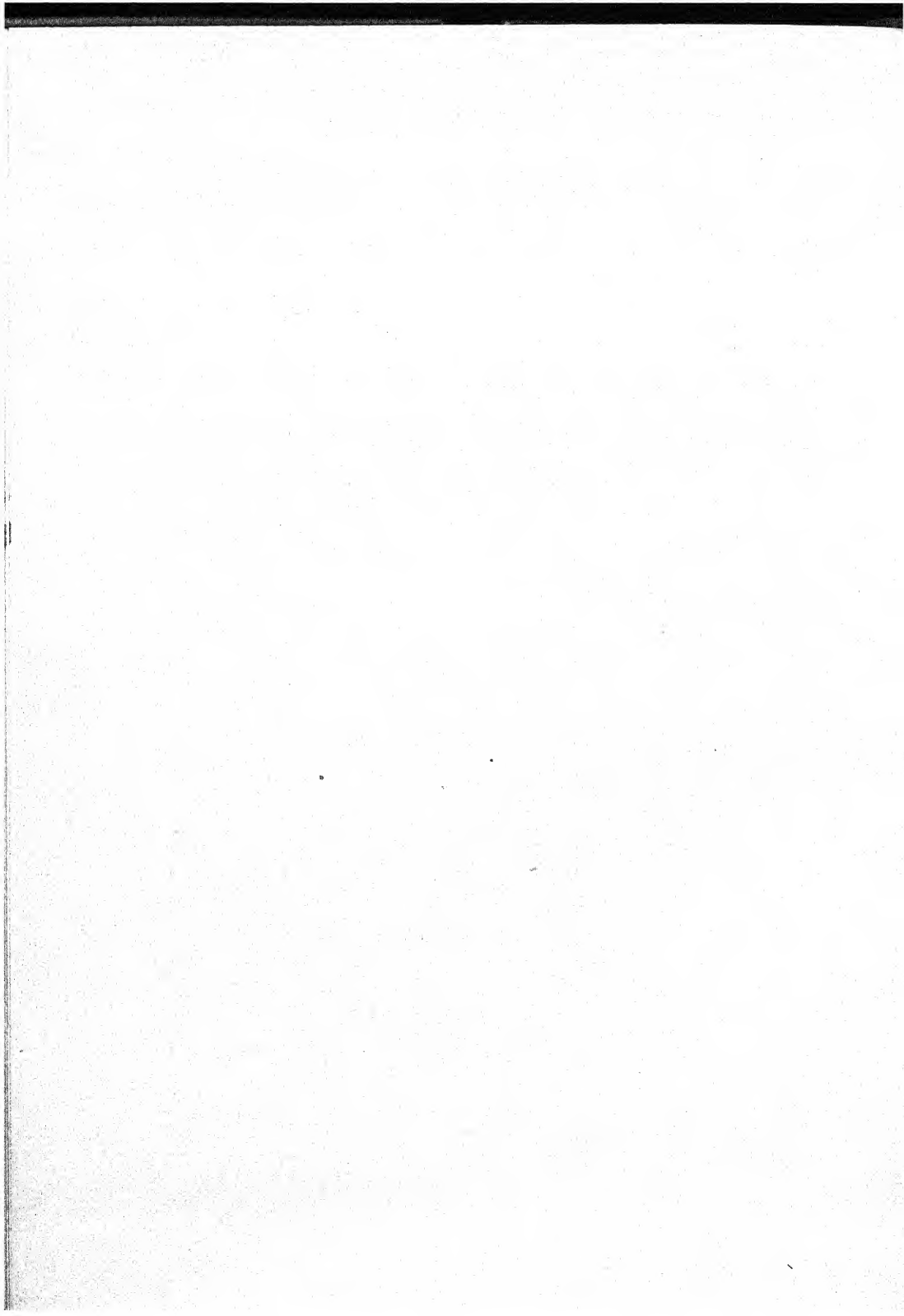
It is natural to wonder how far the later crystallographic work on the sterols was in fact assisted by the knowledge we had by then of the outlines of the sterol skeleton. In crystal structures such as those of the phthalocyanines the crystallographic evidence does provide a direct determination of the molecular structure, which is entirely independent of chemical considerations. In a large part also of the determination of the cholesteryl iodide structure the X-ray evidence is unambiguous; but there are points where experimental difficulties occur and where chemical information is a valuable aid in the crystallographic analysis. Doubtless X-ray studies of new types of molecular and crystal structure will prove to involve additional problems and complications of their own. But whether the crystallographic analysis of a complex asymmetric molecule can be made completely independent of its chemical study is probably largely an irrelevant question since the two methods naturally supplement one another at every stage of the investigation.

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